Synthesis of Pyridine and Pyridinium-based Quinone Methide Precursors: Studies Towards the Realkylation of Aged Acetylcholinesterase

Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Chemistry in the undergraduate colleges of The Ohio State University

by

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ABSTRACT

In the human body, acetylcholinesterase (AChE) is an efficient and essential enzyme that is involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline for use in both the sympathetic and parasympathetic nervous system. Any disturbance in this pathway can be disastrous as AChE is responsible for hydrolysis of 740,000 molecules of ACh per minute. Organophosphorus (OP) nerve-agents such as sarin, soman, and tabun are known to inhibit AChE by forming a covalent bond with the oxygen of the Serine-203 residue located in the active site. Currently, oxime-based pharmaceuticals are available for treatment of the inhibited form of AChE and have been shown to restore function. However, following exposure to OPs, an addition and irreversible process known as "aging" takes place in which the OP-AChE complex experiences the loss of an alkyl group. This process results in the formation of a more stable phospho-anion species that is unaffected by current therapeutics due to the dramatic change in reactivity. On the basis of computational studies, this thesis focuses on the synthesis of small molecules capable of realkylating the aged OP-AChE complex. High energy quinone methides have shown promise as potential therapeutics as previous studies have revealed participation in kinetically favored alkylation of phosphodiesters. We have employed computational chemistry to guide our synthetic efforts and have developed methods to synthesize various quinone methide precusors (QMP); including pyridine and pyridinium compounds. Current results confirm the structure of the neutral pyridine compounds as well as the pyridinium salts by ^{1}H NMR and ^{13}C NMR. Optimization of current synthetic processes as well as testing these compounds as potential re-alkylators or reactivators are in progress.

ACKNOWLEDGEMENTS

I would like to thank my research advisors, Dr. Christopher Callam and Dr. Christopher Hadad for the support they have provided me with over the past year. I have very much enjoyed conducting research as a part of their group and I am grateful for the opportunity I have had to learn from them as well as from the other members of the group.

I would like to thank Dr. Ryan Yoder as well as his undergraduate researchers for their computational work regarding the compounds discussed in this thesis. Throughout this project, computational chemistry has been integral to the design of possible realkylating agents and were essential in guiding the synthesis of these compounds.

I would like to thank Keegan Fitzpatrick, Stephanie Fabry, and Justin Smith for being wonderful lab partners. Their encouragement and friendship has made this experience even more enjoyable and I can only hope to find such great friends as I continue on to graduate school.

Lastly, I would like to thank the Choose Ohio First scholarship program as well as The Ohio State University Department of Chemistry and Biochemistry for providing me with the financial support necessary to be able to pursue research during the academic year.

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1. INTRODUCTION

Organophosphorus (OP) nerve agents include compounds such as sarin, soman, and tabun. First synthesized by Dr. Gerhard Schrader in the early 1930s, these compounds are known to be highly toxic, as even small amounts can be lethal. During the Second World War, a total of 12,000 tonnes of Schrader's "Tabun" was synthesized by the Germans, the majority of which was later seized by the Allies, sparking the beginning of OP nerve-agent and pesticide production in both England and the United State.[1]

While the Germans did not use their newly developed "G series" of chemical weapons, Schrader and his collaborators were able to synthesize more than 2,000 OP compounds by the end of the war and even test them in German military laboratories. Since then, it is estimated that over 50,000 organophosphorus compounds have been created including the more stable "V" series synthesized by British scientists in the late 1950s.[1]

These compounds are of great concern due to their commercial availability as pesticides as well as the ease with which they can be synthesized. Organophosphorus compounds are stable species that can be readily dispersed from bombs, rockets, and spray tanks and have seen increased use as chemical weapons since the 1980s. A notable example of the devastation caused by the use of OP compounds as chemical warfare agents occurred during the Iran-Iraq conflict. In 1988, Iraqi forces proceeded to attack the Kurdish city of Halabja by releasing poisonous gases now thought to include Sarin, as well as the nerve-agent VX. The attack reportedly killed 4,000 – 5,000 people and injured several thousands more, leaving them with various degrees of blindness, lasting respiratory problems, and various cancers.[2]

Organophosphorus compounds are not exclusively used as chemical weapons, but as pesticides and herbicides as well. Many of these compounds are essential to farmers in protecting

their crops from various pests and diseases. Though OP pesticides may offer some improvement over organochlorine compounds such as DDT which were previously used as pesticides, small residues of these OP toxins can remain on the crops after harvest and can be ingested by the public.[3] The World Health Organization estimates that each year there are 3,000,000 cases of OP pesticide poisoning worldwide. Approximately 20% of these cases are fatal.[1]

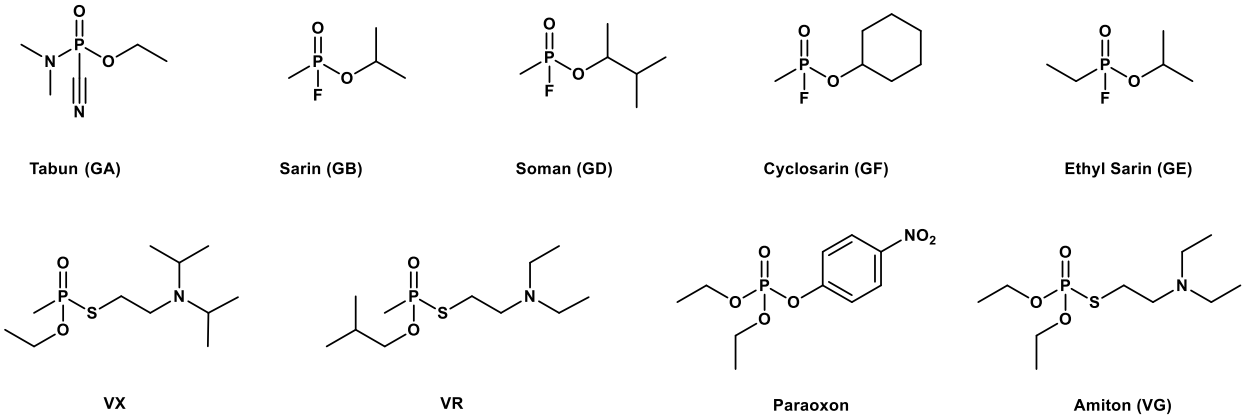
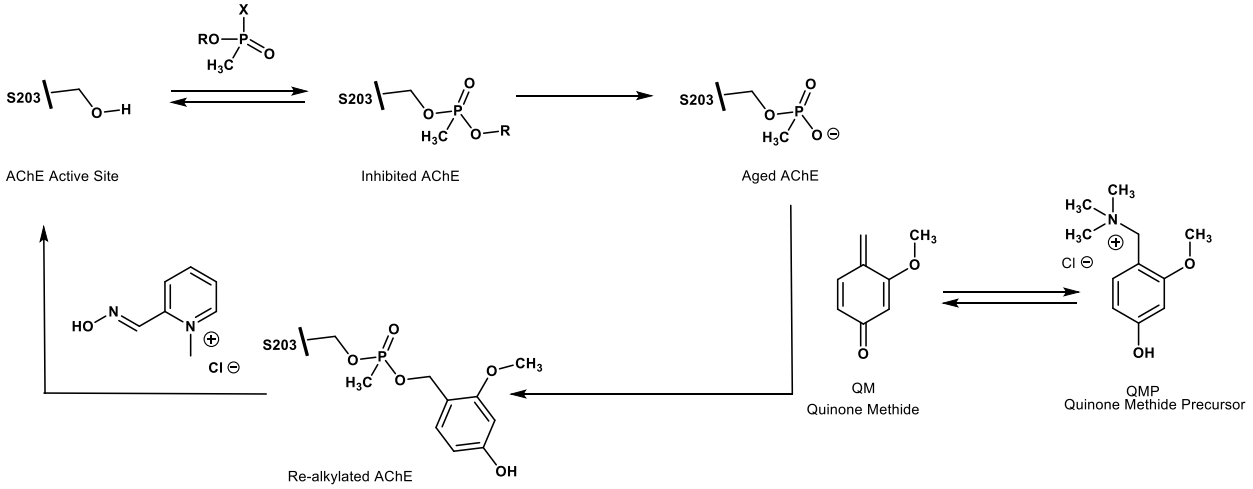


Figure 1.1: OP compounds used as chemical weapons as well as the pesticides "amiton" and "paraoxon".

Organophosphorus (OP) compounds are known covalent inhibitors of the enzyme acetylcholinesterase (AChE). In the body, AChE plays a vital role in both the sympathetic and parasympathetic nervous system as it is responsible for hydrolyzing the neurotransmitter acetylcholine (ACh) into choline for use in biochemical pathways. In order to inhibit the enzyme, the phosphate or phosphonate group of the OP compound participates in the formation of a covalent bond with the Serine-203 residue located in the enzyme active site.[3] This reaction generates what is known as the inhibited form of AChE (Scheme 1.1). Currently, oxime-based pharmaceuticals are available for treatment of the inhibited form of AChE and have been shown to restore the functionality of the enzyme. However, following exposure to OPs, an additional and irreversible process known as "aging" takes place in which the OP-AChE complex experiences

the loss of an alkyl group, leaving a stable methylphosphonate (or phosphate) anion in the active site.



Scheme 1.1: The irreversible aging process of AChE along with the potential reversal via realkylation.

Depending upon which OP compound an individual is affected by, aging of the inhibited enzyme could occur minutes to days later. Any disturbance of the enzyme can be disastrous as AChE is responsible for hydrolysis of 740,000 molecules of ACh per minute.[5] When AChE loses its functionality, acetylcholine begins to rapidly accumulate at the neurosynaptic junctions. The presence of such a large excess of ACh in the brain, blood, and tissue can results in overstimulation as well as partial or complete disruption of a number of pathways in the central nervous system. This can lead to side effects such as convulsions, vomiting, kidney failure, and ultimately death.[2] Once the OP-AChE complex has undergone the aging process, currently available pharmaceuticals no longer have any effect and cannot restore normal AChE functionality to the enzyme.

Quinone methides (QM) are proposed to be essential intermediates in a large number of biological processes[4,6] such as enzyme inhibition, DNA-alkylation and crosslinking. A vast amount of previous research has been conducted on QMs, both *o*-QM and *p*-QM and their reactivity.

Figure 1.2 shows the different structures of QMs that can exist, *o*-QM and *p*-QM are the only closed-shell species for which electrophilic and nucleophilic reactions can occur. The *m*-QM is instead a diradical open shell species. Two of the reasons for the reactivity of QMs are its ability to act as a Michael acceptor as well as the driving force of the structure's return to aromaticity.

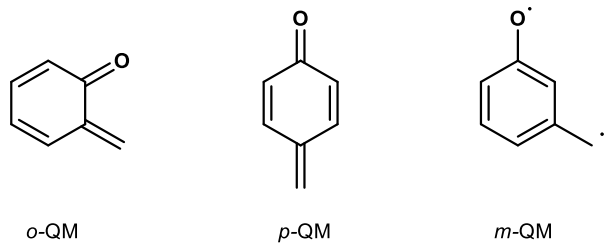
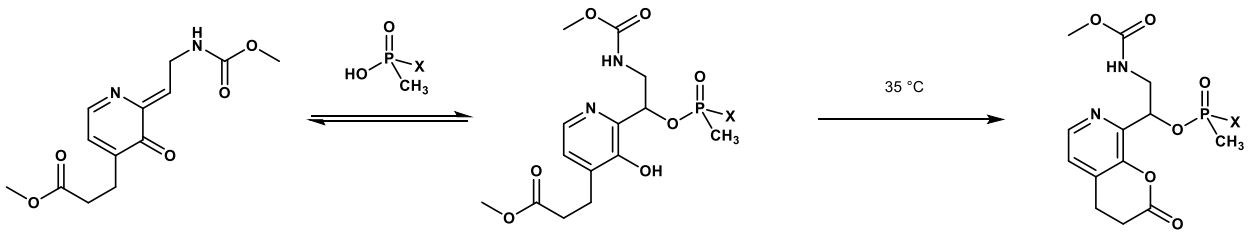


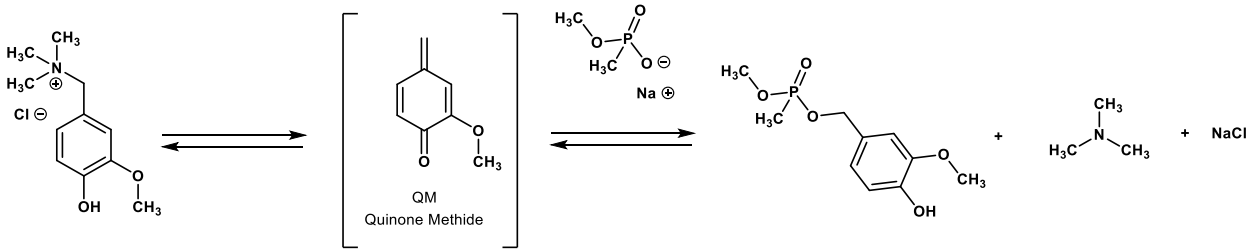
Figure 1.2: General structures of quinone methides (QM).

QMs exhibit electrophilic character at the exocyclic methylene to form benzylic adducts, thus obtaining a new product with an aromatic structure. The electrophilic nature of QMs makes them an attractive intermediate for biological reactions. The documented reactivity of QMs makes them an attractive species for aid in alkylation of phosphodiesters in DNA. Research into the reactivity of *p*-QM shows them successful in alkylation reactions with a phosphodiester, followed by lactonization of the resulting trialkyl phosphate for two functionalized QMs. Shown in Scheme 1.2 is the inspiration for this research which improved efforts to develop a fully functional DNA phosphodiester alkylating agent. The efforts to incorporate the phosphate as an acceptor of an alkyl group is part of an inspiration to attempt to develop an efficient way to reverse the adverse effects of organophosphorus nerve toxins.



Scheme 1.2: Potential favorable substitution and rearomatization drives the reaction of the QM with various organophosphates.

Steinberg has previously tested a library of alkylating agents, mainly haloketones, with *p*-nitrophenyl methylphosphonate, which emulates the aged enzyme.[8] Such haloketones are more reactive to nucleophilic substitution due to the withdrawing nature of the carbonyl. A small library of compounds were selected to be tested with AChE aged with ^{32}P-labeled Soman. The radioactive label was monitored for removal from the active site as a result of reactivation. The library of tested compounds did not show any reactivation of AChE.



Scheme 1.3: Formation of the *p*-QM and subsequent alkylation of the phospho-anion.

Current research has been focused on the synthesis and testing of compounds that have been shown computationally to have the potential to realkylate the aged AChE-OP complex. Due to their electrophilic character and importance in a number of biological processes such as DNA alkylation, linking of structural proteins, and the biosynthesis of lignin in plants, high energy quinone methides (QMs) have been deemed the compounds of interest (Scheme 1.3).[3, 7] A study from Bakke et al. showed the ability of these QMs to realkylate a phosphodiester bond under aqueous conditions.[9] This is encouraging because the OP is believed to participate in a similar phosphodiester bond with Ser-203 before the aging process occurs. This finding, along with the study published by Modica et al. which determined that these QMs had the ability to alkylate

various nucleophiles through the formation of an o-QM intermediate generated from a quinone methide precursor (QMP) is the motivation for some of this research.[10] These findings suggest that *para* and *ortho* QMs could potentially realkylate the aged form of AChE and to allow for the restoration of functionality to the enzyme.

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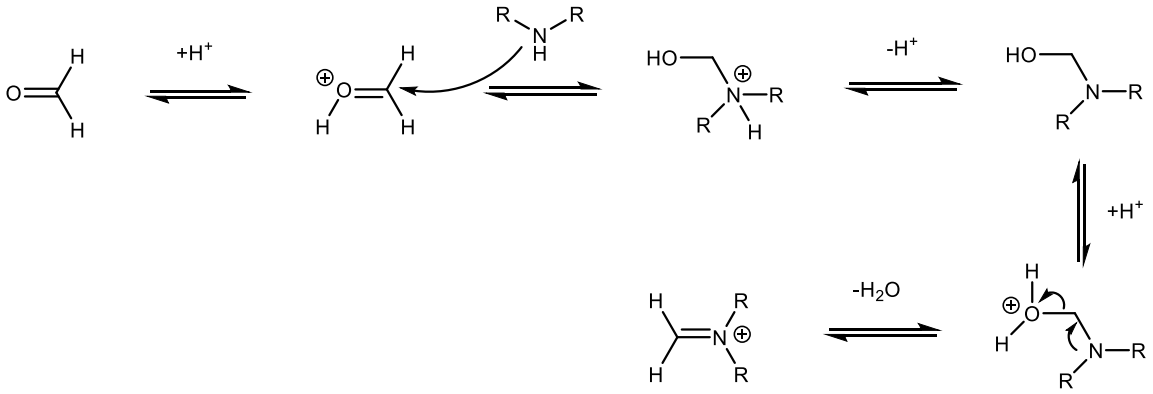
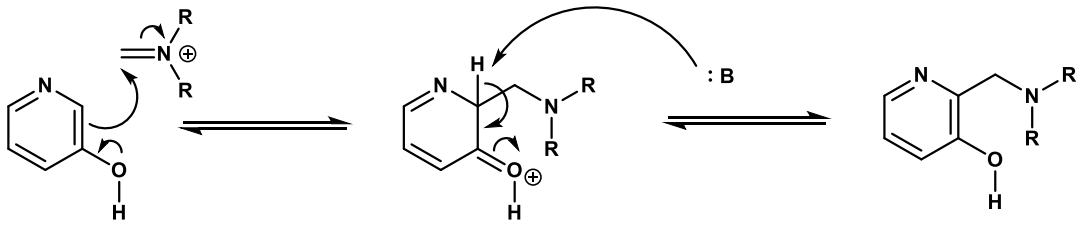
2. RESULTS AND DISCUSSION

2.1 Mannich Reactions. The pyridine based QMPs were synthesized from the commercially available starting material 3-hydroxypyridine in a one-step synthesis, shown in Scheme 2.3. The secondary amine first participates in a Mannich reaction by attacking the formaldehyde to form an electrophile (Scheme 2.1).



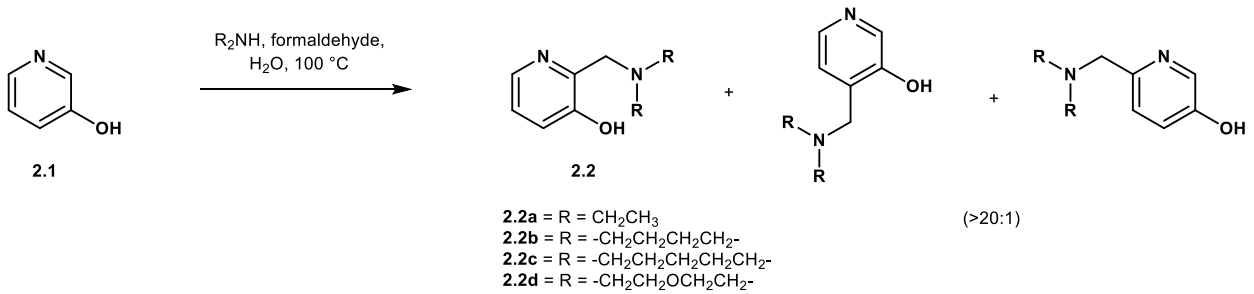
Scheme 2.1: Formation of the iminium salt.

The iminium salt is then attacked by the pyridol ring and the amine group is added to the ring through an electrophilic aromatic substitution pathway (Scheme 2.2). The relatively high yields for these compounds can be seen in Table 2.1. In some cases, the yield was affected by the formation of paraformaldehyde during the reaction period. This was overcome by the addition of excess amine in order to ensure that all formaldehyde present was involved in the formation of the electrophile, thereby preventing the polymer formation.



Scheme 2.2: Addition of the amine group by electrophilic aromatic substitution.

The secondary amine (1 eq) was first allowed to stir in distilled water (5 mL) at room temperature (rt) while the formaldehyde (1eq, 37%) was added. Once Schiff base formation was evident, 3-hydroxypyridine (1 eq) was added and the mixture was allowed to stir until homogenous and was then heated to reflux for 4 hr to produce the neutral pyridine compound.



Scheme 2.3: Mannich Reaction using diethylamine, pyrrolidine, piperidine, and morpholine.

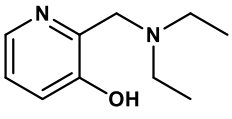
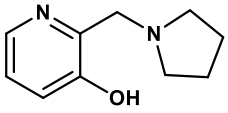
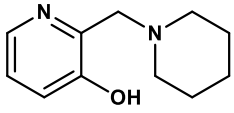
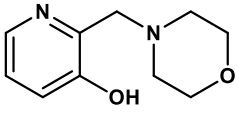
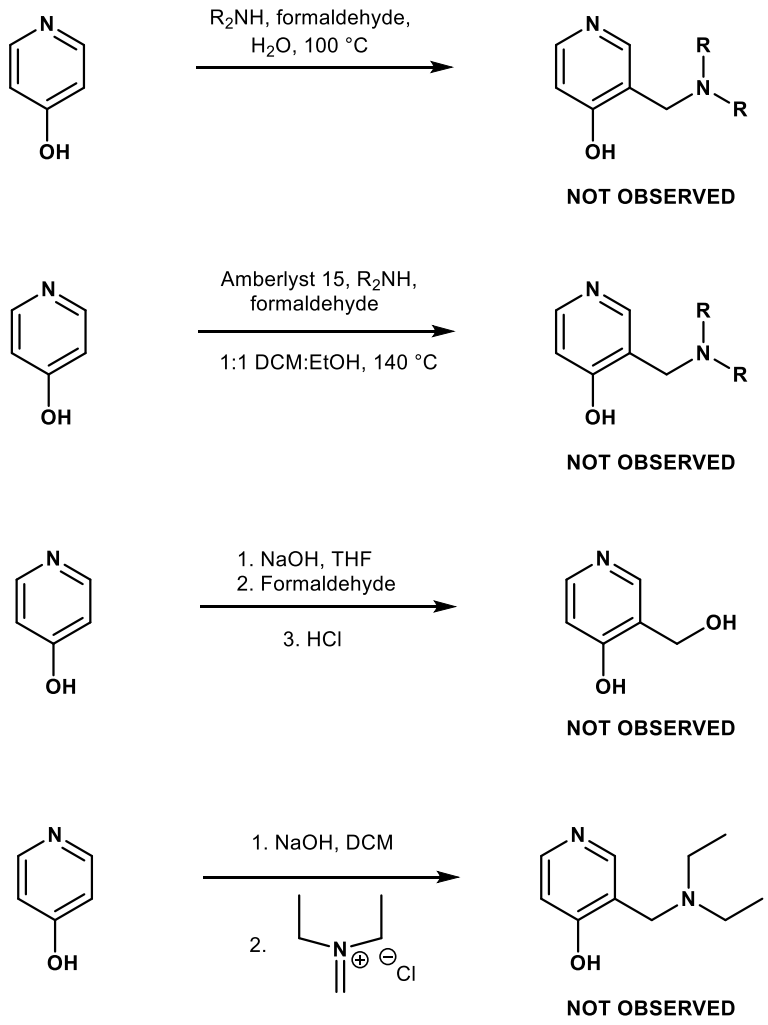
COMPOUNDS				
	2.2a	2.2b	2.2c	2.2d
YIELD	75%	82%	95%	78%

Table 2.1: Neutral pyridine compounds and corresponding yields.

The same reaction was performed on the substrate 4-hydroxypyridine in hopes of further expanding our library of compounds. However, under the same conditions, no product was observed. A number of solvents were explored but ultimately the reaction continued to show no evidence of product formation. An ion exchange catalyst known as Amberlyst 15 was then added to the reaction but also provided no product. After exploring a number of alternative reactions in an attempt to make an addition onto the pyridol ring, the synthesis was deemed unsuccessful (Scheme 2.4).

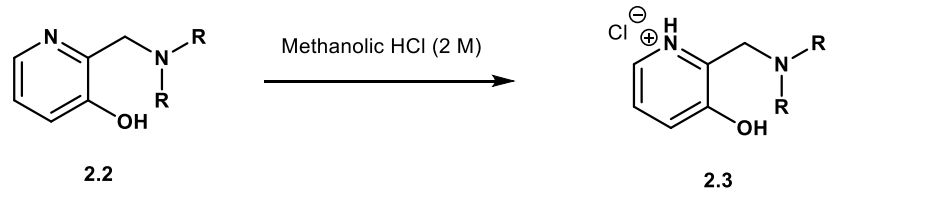


Scheme: 2.4: Reactions explored using the substrate 4-hydroxypyridine.

2.2 Protonation of Neutral Compounds. After the synthesis of the neutral pyridine QMPs via Mannich reactions, the compounds were then protonated to form the pyridinium-based salts. Upon isolating the neutral pyridine compound, the compound is dissolved in toluene (15 mL) before HCl (2M) is added dropwise until the pH of the solution reached 1.

These compounds were previously protonated using an aqueous form of HCl and then characterized by TOF-MS. Upon analysis of the TOF-MS data, it was determined from the m/z ratio that these compounds were indeed gaining only a single proton. In order to reduce the amount of water present in the reaction, methanolic HCl formed by the reaction of acetyl chloride and

methanol has been used instead during the isolation of the salts. Much like the previously discussed Mannich reaction, this reaction provided relatively high yields as seen in Table 2.2.



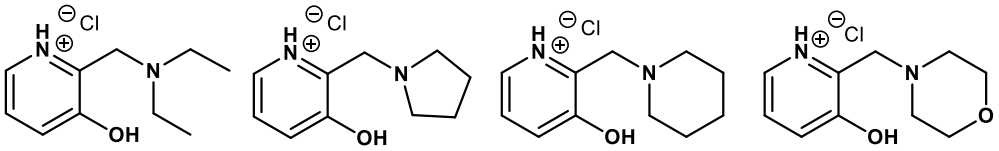
2.2

2.2a = R = CH_2CH_3
2.2b = R = $-CH_2CH_2CH_2CH_2-$
2.2c = R = $-CH_2CH_2CH_2CH_2CH_2-$
2.2d = R = $-CH_2CH_2OCH_2CH_2-$

2.3

2.3a = R = CH_2CH_3
2.3b = R = $-CH_2CH_2CH_2CH_2-$
2.3c = R = $-CH_2CH_2CH_2CH_2CH_2-$
2.3d = R = $-CH_2CH_2OCH_2CH_2-$

Scheme 2.5: Protonation of the neutral pyridine compounds using methanolic HCl.



COMPOUNDS	2.3a	2.3b	2.3c	2.3d
YIELD	88%	90%	87%	99%

Table 2.2: Pyridinium salts and corresponding yields.

Along with the substitution of aqueous HCl with methanolic HCl, characterization via TOF-MS has been substituted by characterization via ^{1}H NMR as well as ^{13}C NMR. Prior to structural analysis via NMR techniques, it was assumed that the proton was adding to form a quaternary ammonium leaving group due to the increased basicity of the amine group relative to the pyridine nitrogen residing in the ring. However, NMR studies have determined that this is not the case and that instead, the proton prefers to reside on the ring nitrogen. This could be due to the potential stabilization of the amine group through participation in a hydrogen bonding interaction with the neighboring hydroxyl group.

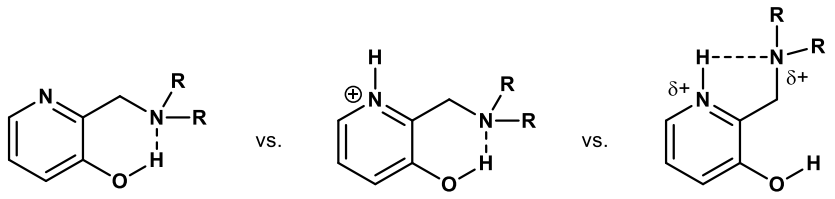
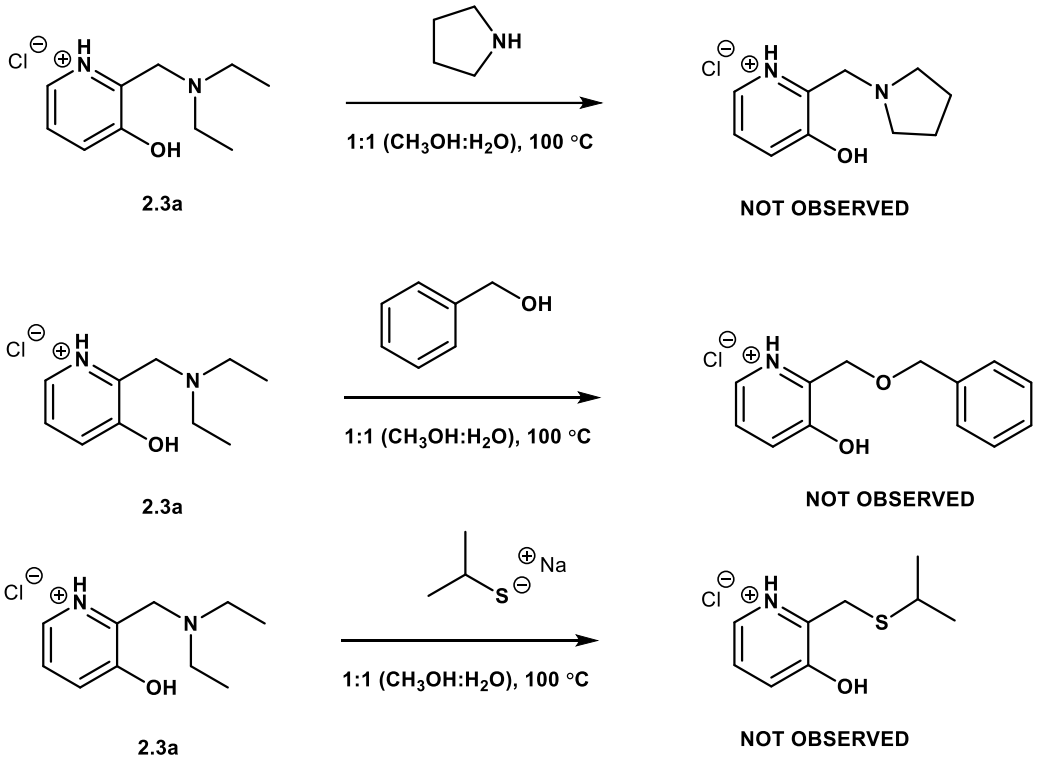


Figure 2.1: Hydrogen bond stabilization of the pyridine and pyridinium QMPs.

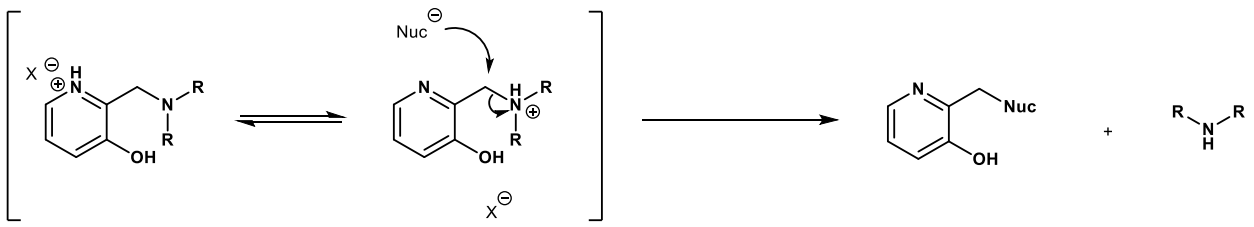
Not only was it determined that the neutral compounds tend to gain a proton on the ring nitrogen, it was also shown that while the hydrogen spends the majority of its time on one nitrogen, in some species, the equilibration of the hydrogen between the two nitrogens can be observed via NMR. This is most prevalent in compound **2.3c** which shows a 3:1 ratio of the two species when CD_3OD is used as the NMR solvent. When the NMR solvent is varied from CD_3OD to DMSO-d_6, the ratio is changed from 3:1 to 10:1 (pK is solvent dependent). This observation acted as the basis for the conclusion that a proton transfer is in fact occurring.

2.3 Nucleophilic Substitution Experiments. Nucleophiles of varying strengths were tested with the pyridinium salts in order to observe any evidence of nucleophilic substitution. Compound **2.3a** was subject to reactions with nitrogen, oxygen, as well as sulfur-based nucleophiles as shown in Scheme 2.6. The reactions were carried out in 1:1 (CH_3OH: H_2O) at 100 °C and were monitored by GC/MS at the time points 1, 4, and 8 hr. Ultimately, the lack of a peak corresponding to product formation on the resulting chromatogram at all time points indicated that nucleophilic substitution was unsuccessful under these conditions. The substitution experiment with 2-propanethiol sodium salt was repeated in toluene in order to allow for a higher reflux temperature. However, these conditions also did not lead to product formation.

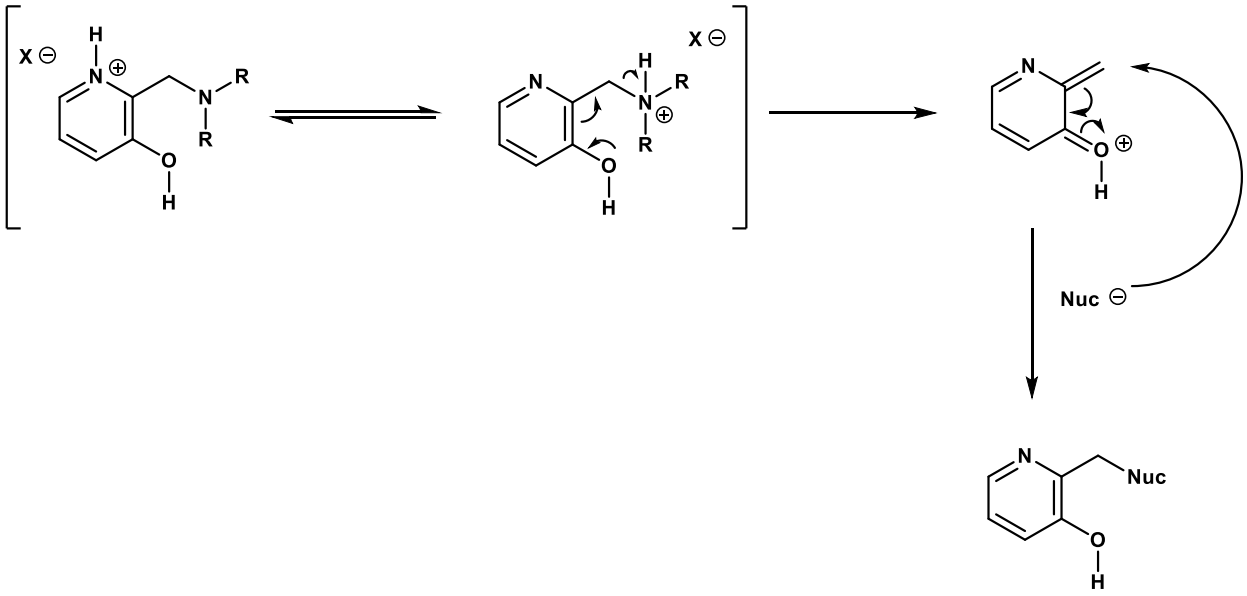


Scheme 2.6: Nucleophilic substitution experiments with pyridinium salts.

2.4 Discussion of Potential Mechanism. While the pyridinium salt **(2.3a)** tested did not undergo nucleophilic substitution when subjected to various nucleophiles, this does not necessarily mean that it will not do so in the active site of AChE as the interior environment of an enzyme is far different from any reaction vessel. At this time, two mechanisms of displacement have been proposed including nucleophilic substitution via an S_N2 displacement as seen in Scheme 2.7, as well as nucleophilic substitution by way of a high energy QM intermediate as shown in Scheme 2.8.

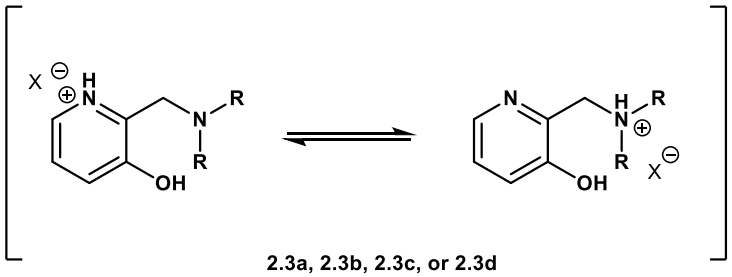


Scheme 2.7: Nucleophilic substitution via the proposed S_N2 displacement mechanism.



Scheme 2.8: Nucleophilic substitution via high energy QM intermediate.

In order for a compound to proceed through either mechanism, the quaternary ammonium cation must act as a leaving group. As previously discussed, a number of the pyridinium salts have shown the existence of an equilibrium between two isomers (Scheme 2.9) by NMR studies.



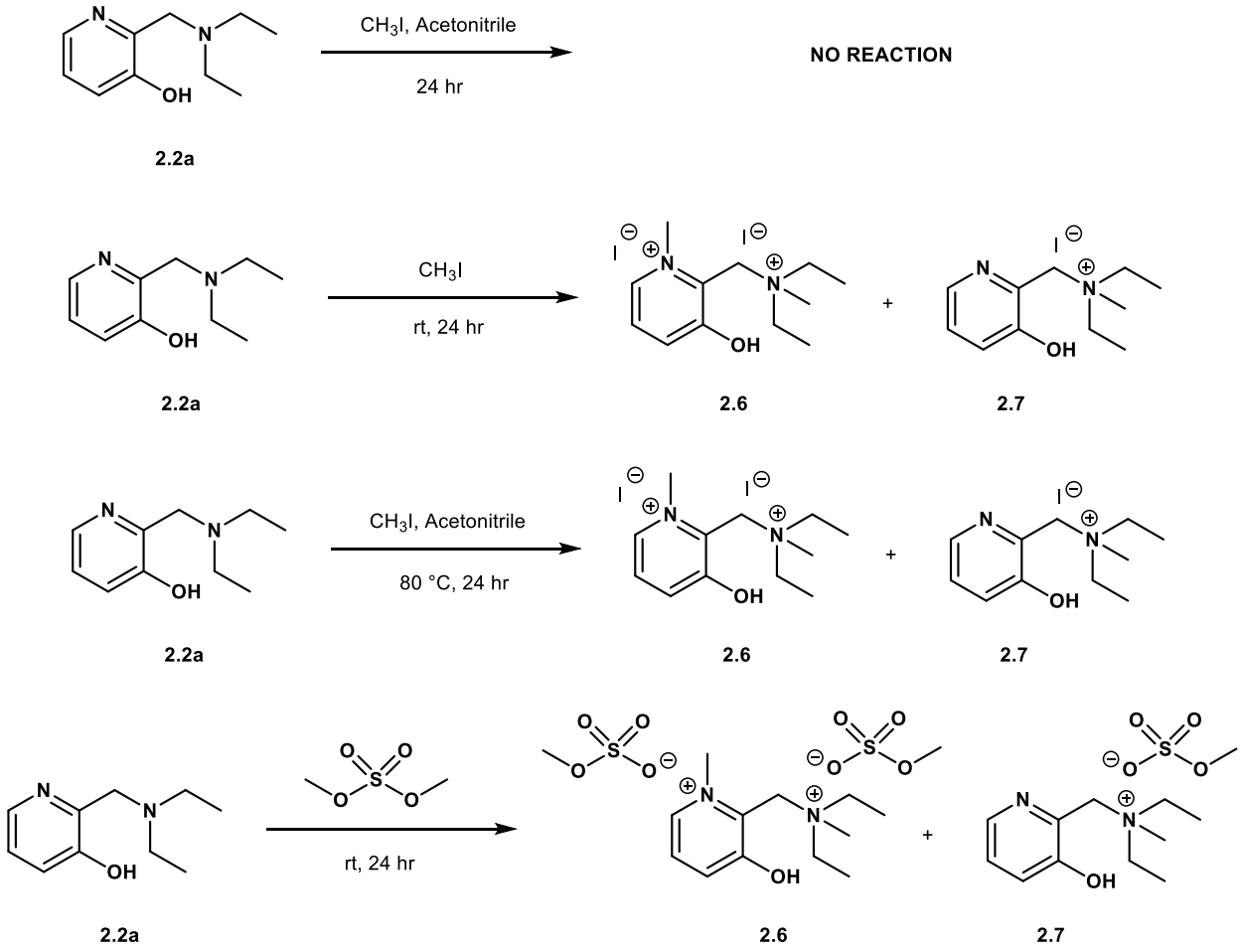
Scheme 2.9: Equilibrium between two isomers of select pyridinium-based salts.

Those that did not show the presence of this equilibrium, preferred to become protonated at only the ring nitrogen. Compound **2.3c** showed the highest of the observed equilibrium ratios with a 3:1 ratio existing between the two isomers. Again, favoring the acidic proton residing on the ring nitrogen. Therefore, regarding this family of pyridine and pyridinium-based compounds, the prerequisite leaving group for either displacement event to occur is present only one third of

the time at most. In order to determine if displacement is occurring and which of the proposed pathways it may be occurring by, additional mechanistic probing studies are necessary including enzyme kinetic studies.

3. CURRENT AND FUTURE WORK

3.1 Methylation Reactions. In order to continue to expand our QMP library, methylation of the neutral pyridine-based QMPs has been investigated. Various methylating agents as well as reaction conditions have been incorporated (Scheme 3.1) in hopes of producing either a mono or di-methylated product.

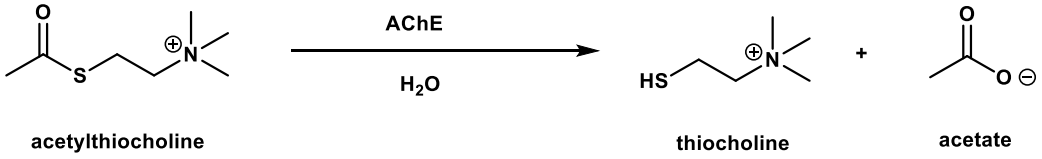


Scheme 3.1: Methylation of neutral pyridine-based compounds.

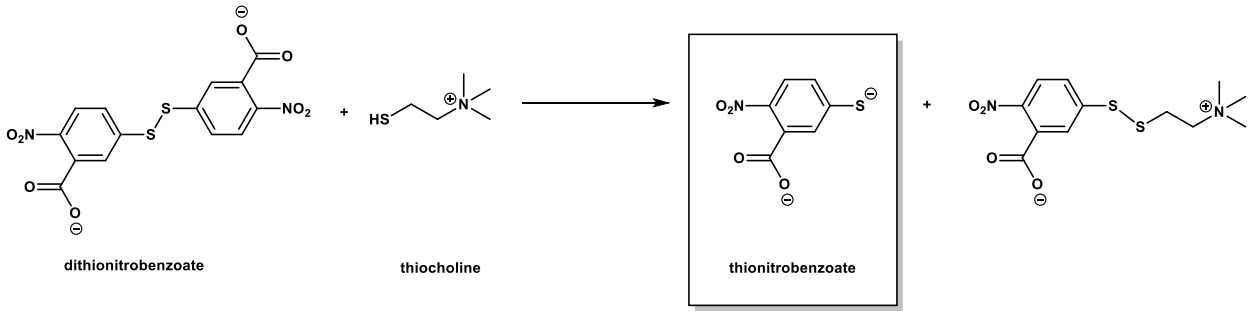
First, the neutral compound **(2.2a)** was dissolved in acetonitrile and the methylating agent methyl iodide was added. The reaction was allowed to stir for 24 hr. At the end of the reaction period no product was observed. The reaction was then repeated without the presence of solvent. After stirring for 24 hr, product formation was evident, however, the crude product was shown to

be a mixture of the mono and di methylated products which could not be separated. This trend of mixed products continued even as more harsh methylating agents such as dimethyl sulfate were investigated.

3.2 Kinetic Studies. Ellman's Assay has been used to monitor the activity of both AChE as well as aged AChE during and after the exposure to a number of compounds in our current library. In this assay, acetylcholinesterase from *Electrophorus electricus* (electric eel) is allowed to act on the thioester acetylthiocholine instead of the oxyester acetylcholine. Upon the hydrolysis of the substrate by AChE (Scheme 3.2), the thiocholine generated is able to cleave the disulfide bond present in dithionitrobenzoate to liberate thionitrobenzoate (Scheme 3.3), a species which is known to absorb at 412 nm.



Scheme 3.2: Hydrolysis of acetylthiocholine to choline by AChE.



Scheme 3.3: Cleavage of the disulfide bond of DTNB resulting in the production of a species capable of absorption at 412 nm.

In this way, activity of the enzyme can be quantified by monitoring the absorbance at 412 nm via UV-Vis spectroscopy. By quantifying the enzyme activity before and after exposure to various

concentrations of these pyridine and pyridinium-based QMPs, the interaction between QMP and enzyme can be investigated by looking for any sign of enzyme inhibition.

Enzyme inhibition itself can occur by many different mechanisms. However, in order to be in the ideal position for realkylation of the aged AChE-OP complex, the compound must be in close proximity to the enzyme active site. Propidium iodide is a well-known reagent often used for the staining of dead cells and has also shown to be an inhibitor of AChE through binding to the peripheral anionic site (PAS). When bound to the PAS, propidium is known to fluoresce. While it is difficult to prove that these QMPs are in fact inhibiting the enzyme through binding in the active site, an Ellman's type assay performed in the presence of propidium iodide could potentially rule out the possibility that these compounds are inhibiting at the PAS as the measured fluorescence would change as compared to a control if this were the case.

4. EXPERIMENTAL

4.1 General. All solvents used did not undergo any further purification. The reactions performed were carried out at standard atmospheric pressure and were routinely monitored via TLC on silica gel 60 F254 (0.25 mm, E. Merck). Compounds were detected with the use of a UV lamp as well as by either phosphomolybdic acid (PMA) stain or potassium permanganate ($KMNO_4$) stain followed by the addition of heat. The drying agent used after each extraction step was anhydrous Na_2SO_4. Characterization of products was done using both ^{1}H NMR and ^{13}C NMR. Spectra collected via ^{1}H NMR were recorded at 400 MHz and any chemical shifts were referenced to 7.27 for $CDCl_3$. Regarding spectra collected using ^{13}C NMR, chemical shifts were referenced to 77.00 for $CDCl_3$ and data was recorded at 100.6 MHz.

4.2 General Procedure for Mannich Reactions

Method A. Diethylamine (1.090 mL, 10.542 mmol, 1 eq.) was stirred in distilled water (5 mL) while formalin (0.785 mL, 10.542 mmol, 37%, 1 eq.) was added to the reaction vessel. Once formation of the Schiff base was evident, 3-hydroxypyridine (1.0049 g, 10.567 mmol, 1 eq.) was added and allowed to stir until homogenous. The reaction was heated to reflux for 4 hr and then allowed to cool to rt before the addition of distilled water (30 mL). The solution was stirred and then extracted with dichloromethane (3 x 40 mL). The organic layers were collected and dried using anhydrous Na_2SO_4 and the solvent was evaporated in vacuo at 30 °C to yield **2.2a** as a brown syrup (1.52 g, 8.43 mmols, 75%). **(2.2a):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 7.85 -7.78 (d, 1 H, J = 3.2 Hz), 7.15 -7.12 (dd, 1 H, J = 8.4 Hz, 8.4 Hz), 7.08 -7.07 (d, 1 H, J = 1.2 Hz), 3.94 (s, 2 H), 2.73 -2.65 (m, 4 H), 1.17 -1.01 (m, 6 H); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 155.6, 142.9, 138.3, 123.7, 123.4, 58.0, 46.9, 10.1.

Method B. Pyrrolidine (0.880 mL, 10.542 mmol, 1 eq.) was stirred in distilled water (5 mL) while formalin (0.785 mL, 10.542 mmol, 37%, 1 eq.) was added to the reaction vessel. Once formation of the Schiff base was evident, 3-hydroxypyridine (1.0010 g, 10.526 mmol, 1 eq.) was added and allowed to stir until homogenous. The reaction was heated to reflux for 4 hr and then allowed to cool to rt before the addition of distilled water (30 mL). The solution was stirred and then extracted with dichloromethane (3 x 40 mL). The organic layers were collected and dried using anhydrous Na_2SO_4 and the solvent was evaporated in vacuo at 30 °C to yield **2.2b** as a dark red syrup (1.53 g, 8.58 mmols, 82%). **(2.2b):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 7.84 – 7.83 (d, 1 H, J = 4.4 Hz), 7.16 -7.08 (m, 2 H), 3.99 (s, 2 H) 2.73 – 2.72 (m, 4 H), 1.87 -1.84 (m, 4 H); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 155.4, 143.0, 138.1, 123.9, 123.4, 60.4, 53.3, 23.2.

Method C. Piperidine (1.040 mL, 10.528 mmol, 1 eq.) was stirred in distilled water (5 mL) while formalin (0.785 mL, 10.542 mmol, 37%, 1 eq.) was added to the reaction vessel. Once formation of the Schiff base was evident, 3-hydroxypyridine (0.9401g, 9.8853 mmol, 1 eq.) was added and allowed to stir until homogenous. The reaction was heated to reflux for 4 hr and then allowed to cool to rt before the addition of distilled water (30 mL). The solution was stirred and then extracted with dichloromethane (3 x 40 mL). The organic layers were collected and dried using anhydrous Na_2SO_4 and the solvent was evaporated in vacuo at 30 °C to yield **2.2c** as a yellow syrup (1.81 g, 9.41 mmols, 95%). **(2.2c):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 7.92 -7.91 (d, 1 H), 7.19 -7.11 (m, 2 H) 3.86 (s, 2 H), 2.63 -2.57 (m, 4 H), 1.67 -1.58 (m, 4 H), 1.57 -1.51 (m, 2 H); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 155.0, 142.5, 138.7, 123.7, 123.2, 62.9, 53.7, 25.5, 23.9.

Method D. Morpholine (0.920 mL, 10.518 mmol, 1 eq.) was stirred in distilled water (5 mL) while formalin (0.785 mL, 10.542 mmol, 37%, 1 eq.) was added to the reaction vessel. Once formation of the Schiff base was evident, 3-hydroxypyridine (0.9936 g, 10.448 mmol, 1 eq.) was added and

allowed to stir until homogenous. The reaction was heated to reflux for 4 hr and then allowed to cool to rt before the addition of distilled water (30 mL). The solution was stirred and then extracted with dichloromethane (3 x 40 mL). The organic layers were collected and dried using anhydrous Na_2SO_4 and the solvent was evaporated in vacuo at 30 °C to yield **2.2d** a yellow-orange solid (1.59 g, 8.19 mmols, 78%). **(2.2d):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 7.55 – 7.54 (dd, 1 H, *J* = 4 Hz, 4 Hz), 6.81 -6.72 (m, 2 H, *J* = 33.6 Hz), 3.45 (s, 2 H), 3.31 -3.29 (t, 4 H, *J* = 9.6 Hz), 2.175 -2.153 (t, 4 H, *J* = 8.8 Hz); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 154.2, 142.3, 139.2, 123.9, 123.3, 66.3, 62.0, 52.9.

4.3 General Procedure for Protonation of Neutral Compounds

Method A. Toluene (15 mL) was added to **2.2a** (0.210 g, 1.165 mmols) and the solution was stirred until homogenous. Methanolic HCl (approx. 2M) was added dropwise to the solution until a pH of 1 was reached. Once pH 1 was achieved, the solution stirred for an additional 1 hr before the solvent was evaporated in vacuo at 60 °C to yield **2.3a** as a tan crystalline solid (0.2213 g, 1.0212 mmols, 88%). **(2.3a):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 8.31 -8.29 (d, 1 H, *J* = 5.7 Hz), 7.95 -7.92 (d, 1 H, *J* = 8.8 Hz), 7.84 -7.82 (d, 1 H, *J* = 8 Hz), 4.56 (s, 2 H), 3.25 -3.18 (m, 4 H), 1.28 -1.20 (m, 6 H); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 156.6, 134.6, 132.2, 131.7, 129.2, 48.5, 48.2, 7.8.

Method B. Toluene (15 mL) was added to **2.2b** (0.5042 g, 2.8288 mmols) and the solution was stirred until homogenous. Methanolic HCl (approx. 2M) was added dropwise to the solution until a pH of 1 was reached. Once pH 1 was achieved, the solution stirred for an additional 1 hr before the solvent was evaporated in vacuo at 60 °C to yield **2.3b** as a tan crystalline solid (0.5452 g, 2.5395 mmols, 90%). **(2.3b):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 8.47 -8.46 (d, 1 H, *J* = 5.2 Hz),

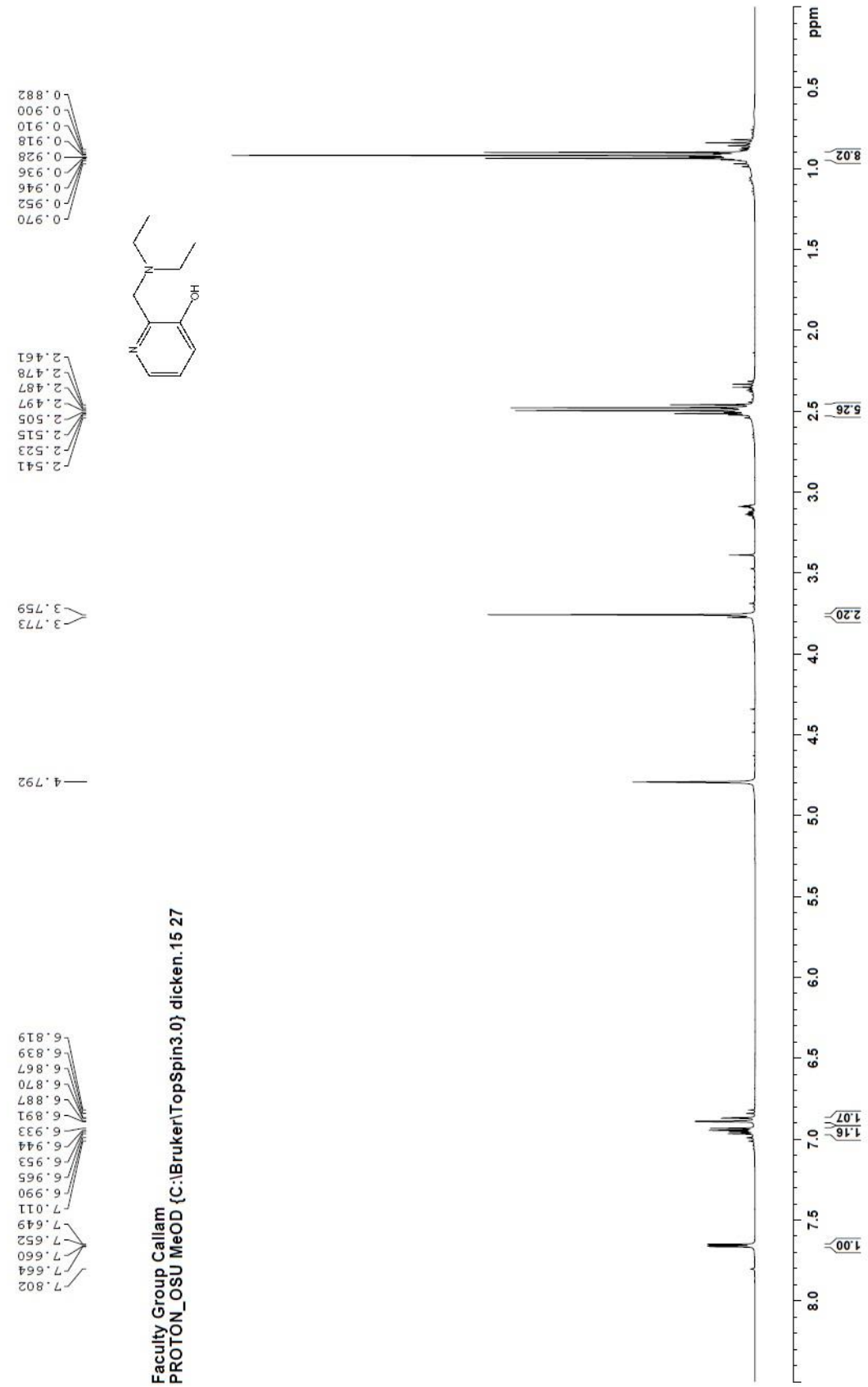
8.13 -8.00 (dd, 2 H, *J* = 5.2 Hz, 8.8 Hz), 4.73 (s, 2 H), 3.42 -3.36 (m, 4 H), 1.45 -1.37 (m, 4 H); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 156.6, 134.6, 132.2, 131.70, 129.2, 48.5, 48.2, 7.8.

Method C. Toluene (15 mL) was added to **2.2c** (0.200 g, 1.040 mmols) and the solution was stirred until homogenous. Methanolic HCl (approx. 2M) was added dropwise to the solution until a pH of 1 was reached. Once pH 1 was achieved, the solution stirred for an additional 1 hr before the solvent was evaporated in vacuo at 60 °C to yield **2.3c** as a tan crystalline solid (0.2051 g, 0.9007 mmols, 87%). **(2.3c):** ^{1}H NMR (400 MHz, CD_3OD, δ_H) 8.44 -8.43 (d, 1 H, *J* = 5.2 Hz), 8.13 – 8.11 (d, 1 H, *J* = 8.8 Hz), 8.00 -7.96 (dd, 1 H, *J* = 8.8 Hz, 5.2 Hz), 4.65 (s, 2 H), 3.27 -3.01 (m, 4 H), 1.76 -1.73 (m, 4 H), 1.54 – 1.49 (m, 2 H); ^{13}C NMR (100.6 MHz, CD_3OD, δ_C) 157.2, 134.1, 133.3, 130.5, 129.5, 54.0, 51.4, 22.8, 20.8.

Method D. Toluene (15 mL) was added to **2.2d** (0.800 g, 4.119 mmols) and the solution was stirred until homogenous. Methanolic HCl (approx. 2M) was added dropwise to the solution until a pH of 1 was reached. Once pH 1 was achieved, the solution stirred for an additional 1 hr before the solvent was evaporated in vacuo at 60 °C to yield **2.3d** as a tan crystalline solid (0.9388 g, 4.0695 mmols, 99%). **(2.3d):** ^{1}H NMR (400 MHz, D_2O, δ_H) 8.36 -8.35 (d, 1 H, *J* = 4 Hz), 8.00 -7.98 (d, 1 H, *J* = 8 Hz), 7.89 -7.85 (m, 1 H), 4.69 (s, 2 H), 4.13 -3.92 (m, 4 H), 3.57 -3.51 (m, 4 H); ^{13}C NMR (100.6 MHz, D_2O, δ_C) 155.8, 136.3, 131.8, 130.7, 129.2, 63.6, 53.4.

APPENDIX A

^{1}H NMR and ^{13}C NMR Spectra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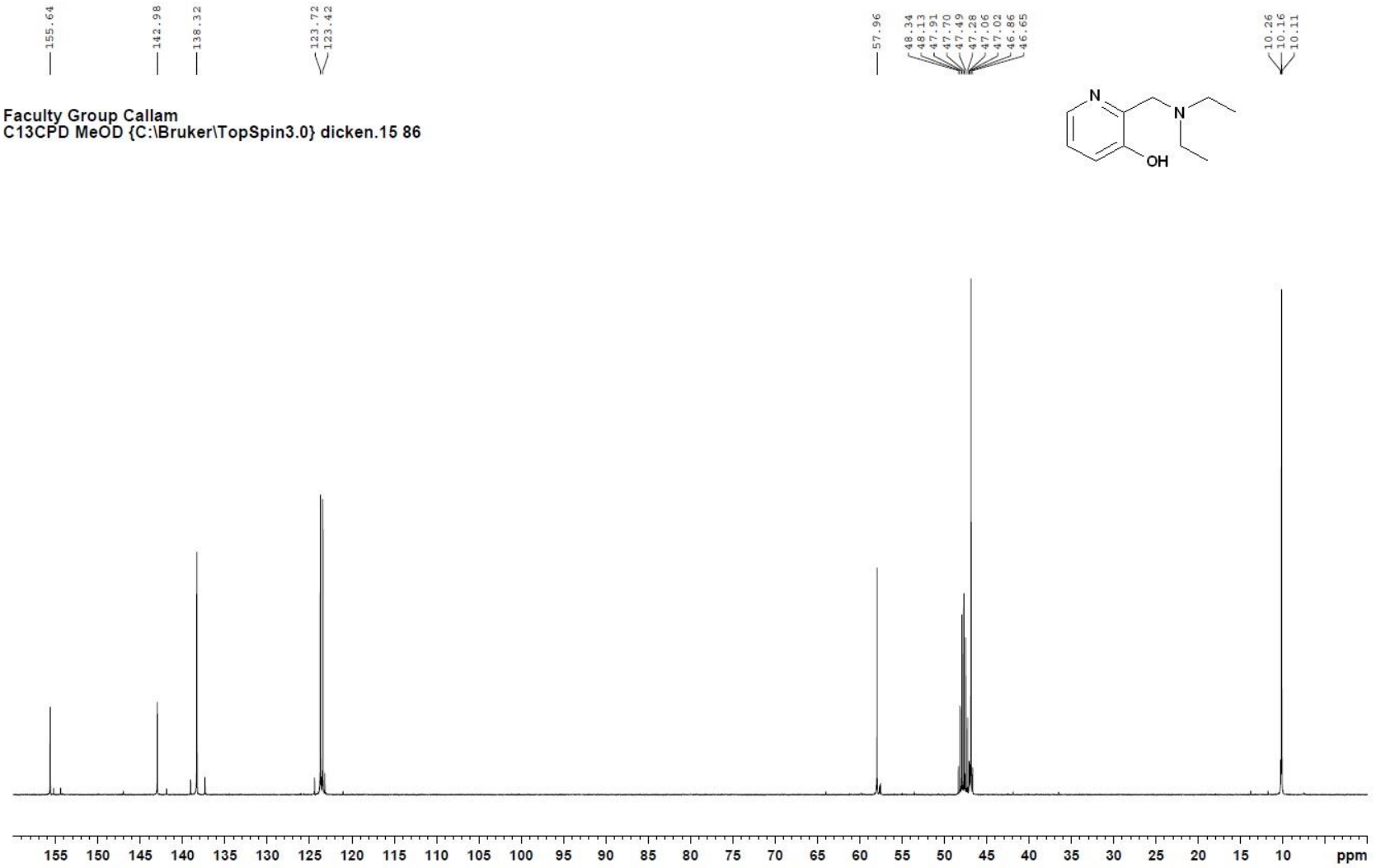
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46.65
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10.16
10.11
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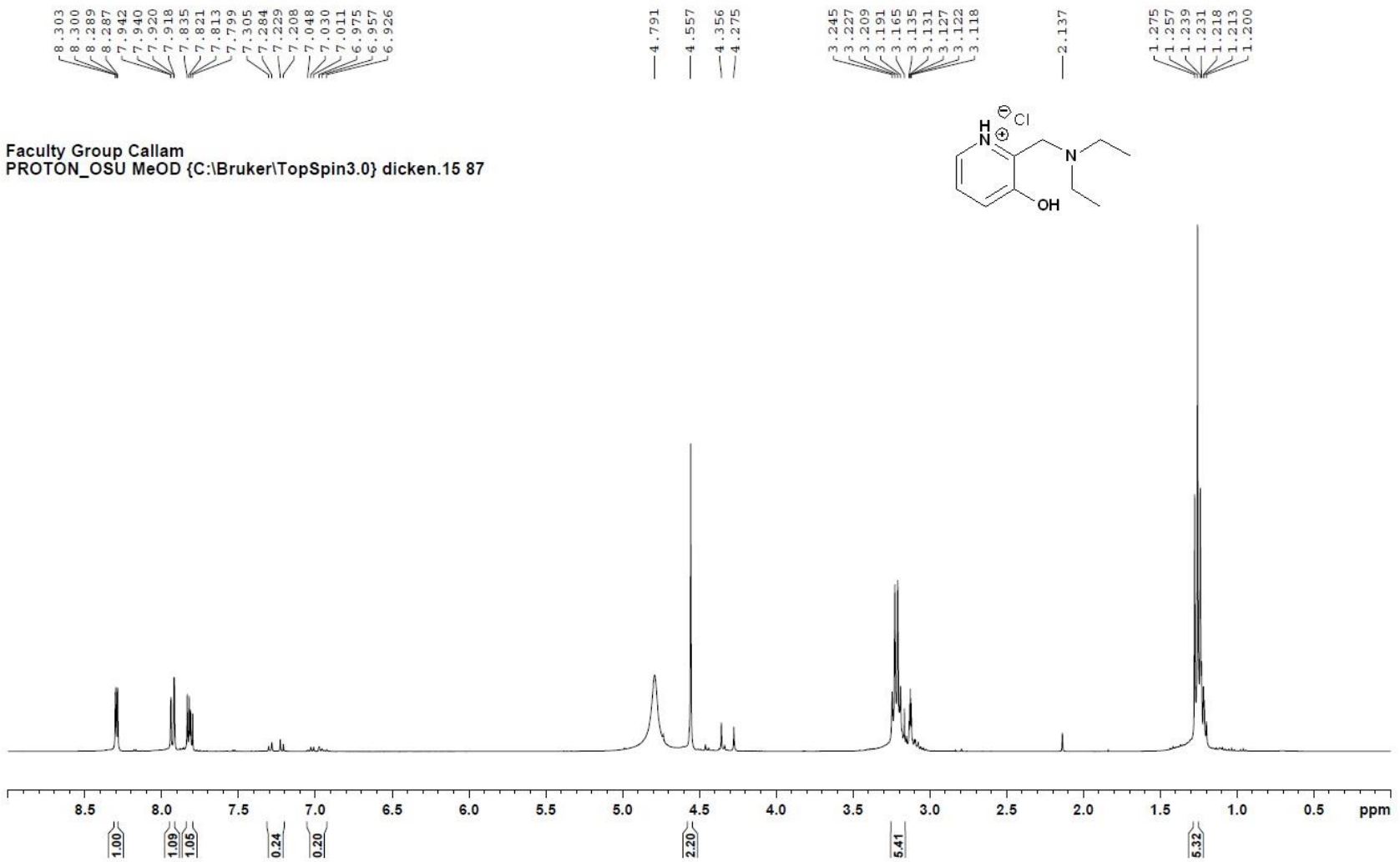
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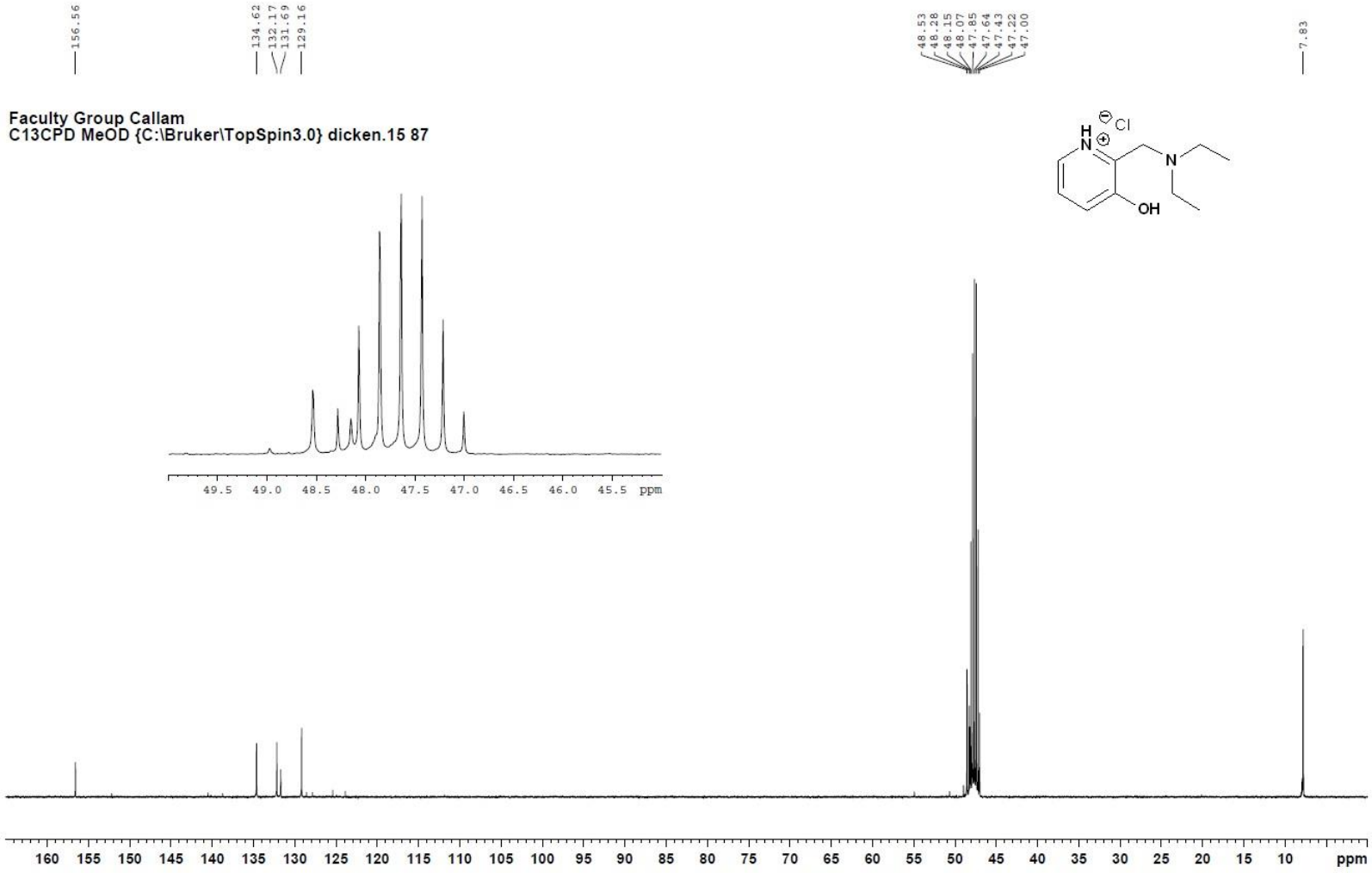
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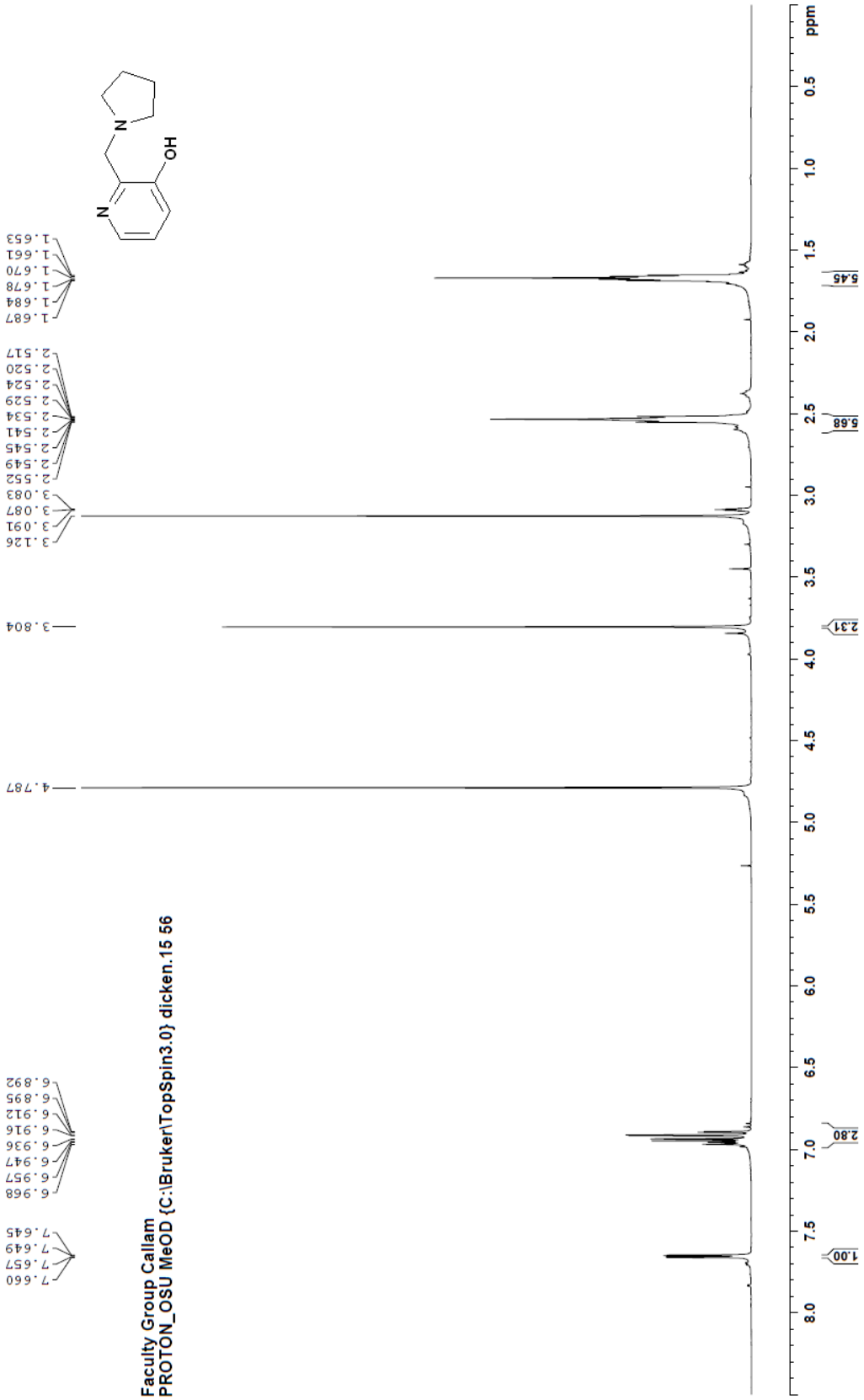
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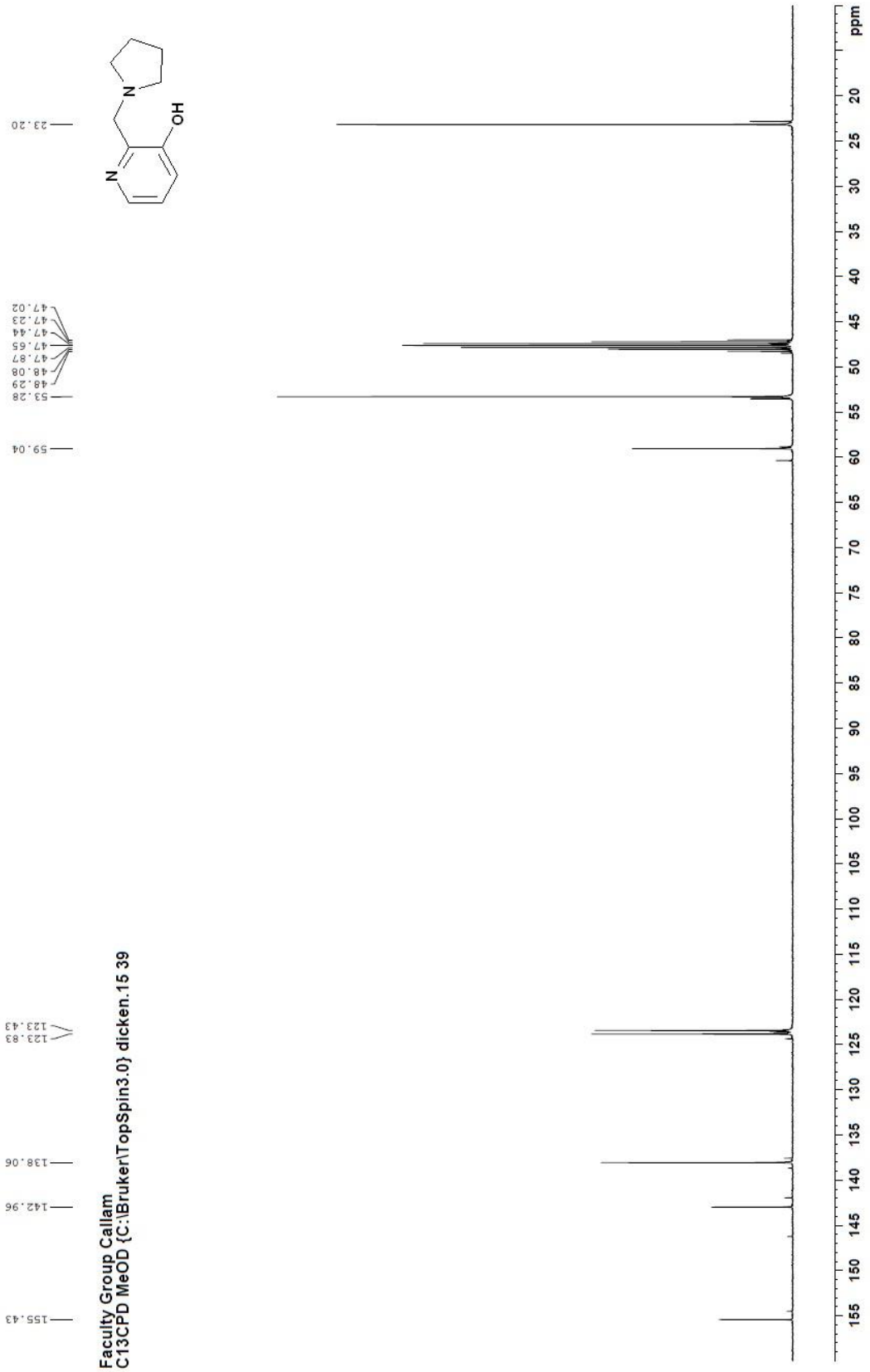
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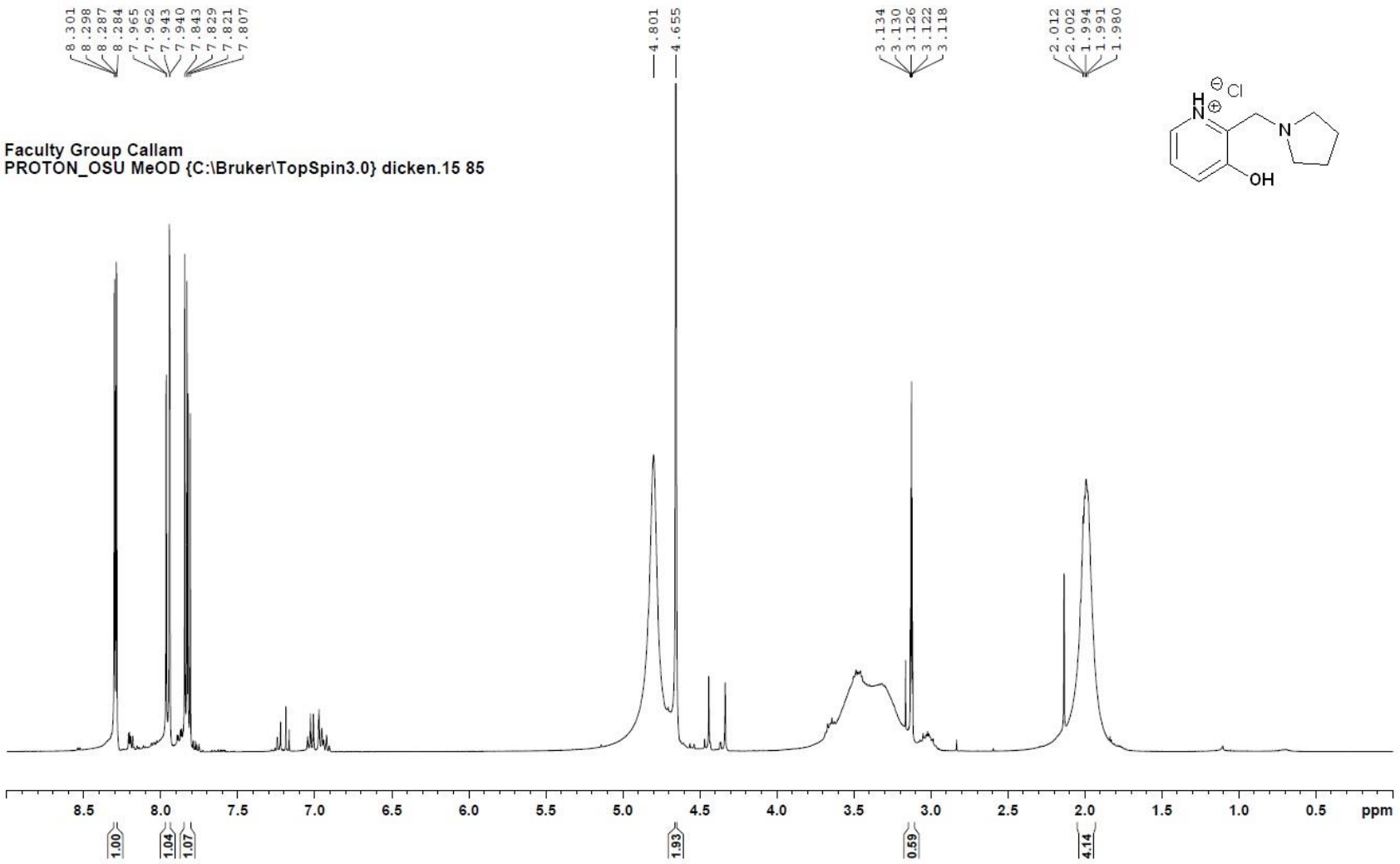
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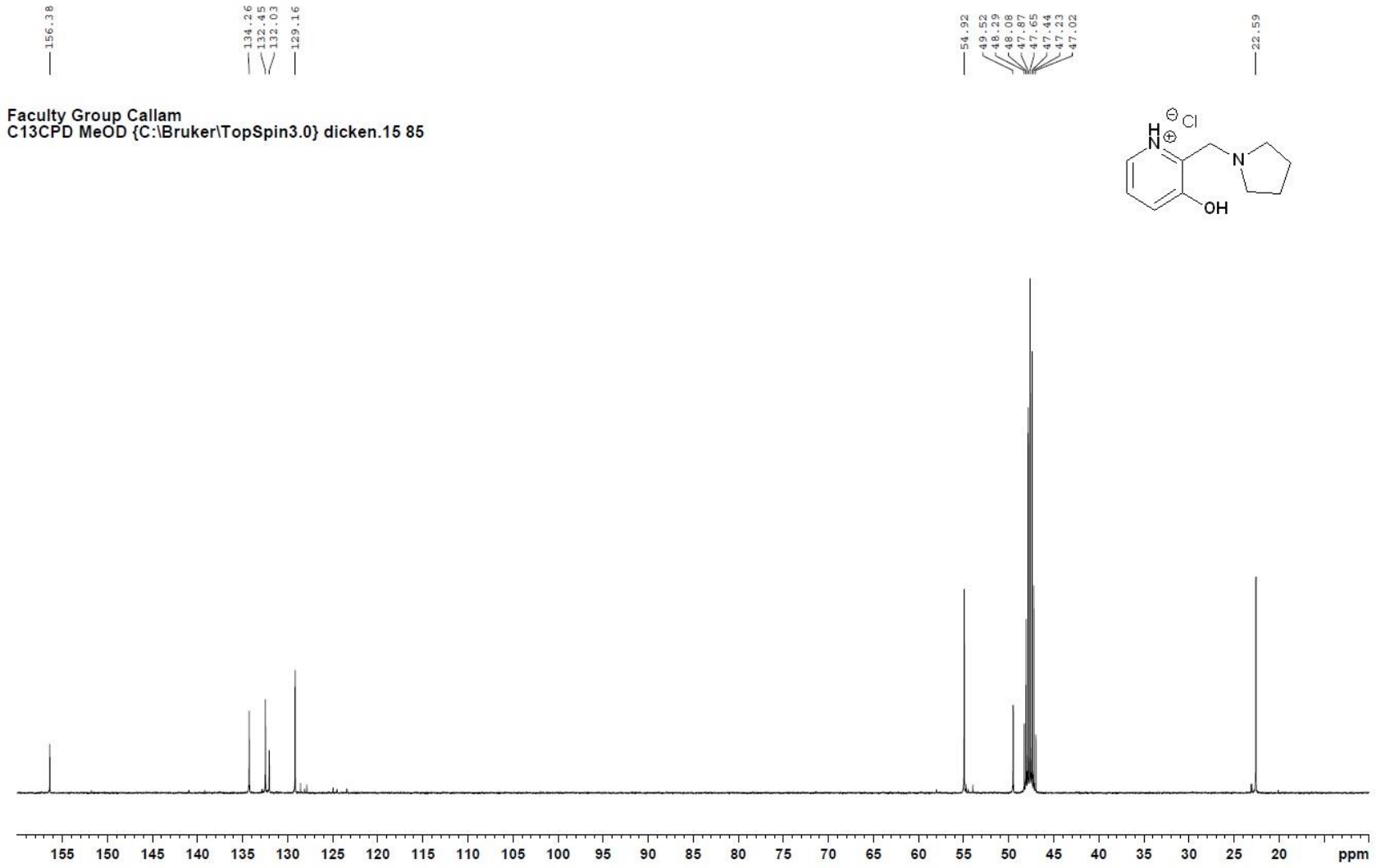
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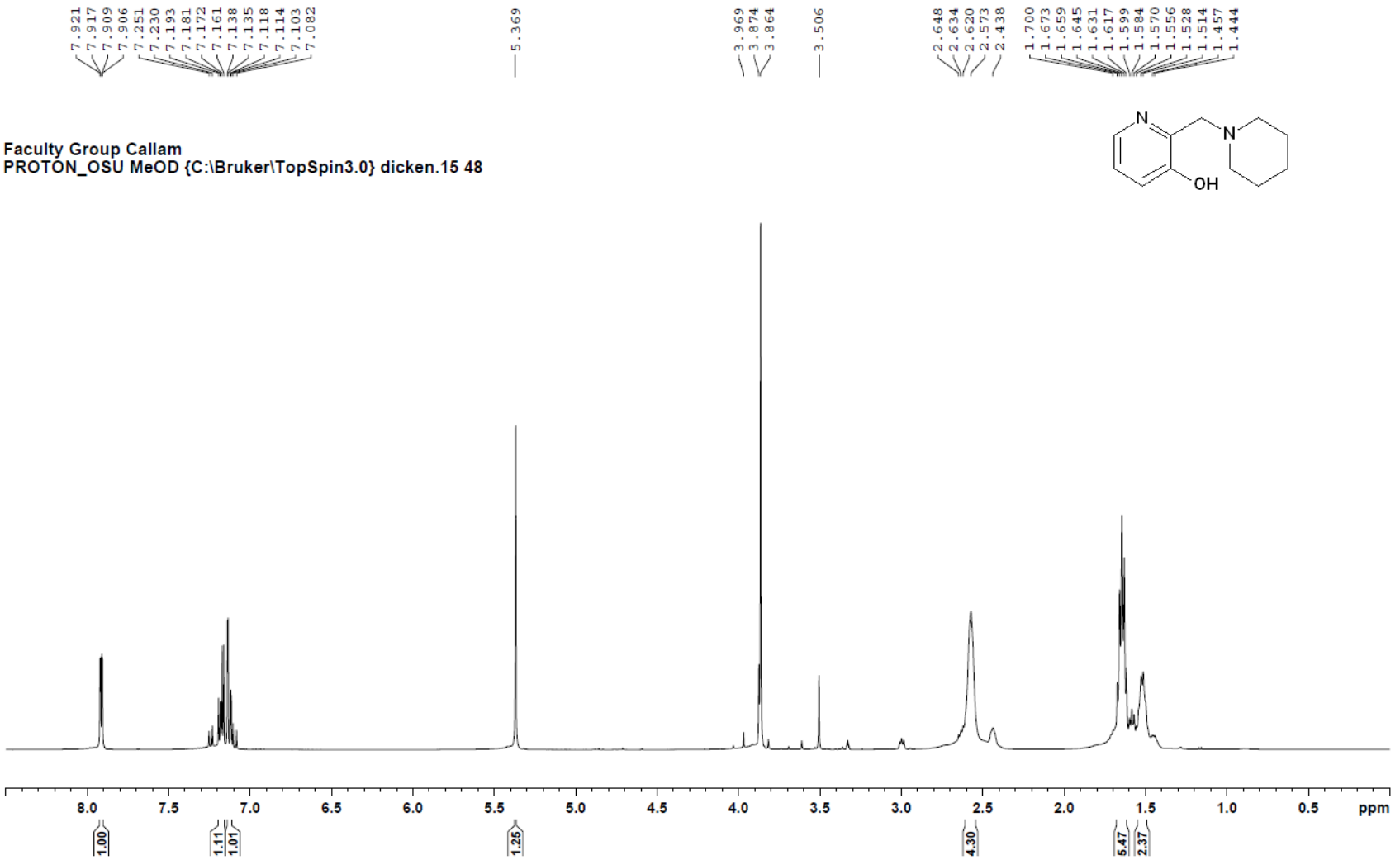
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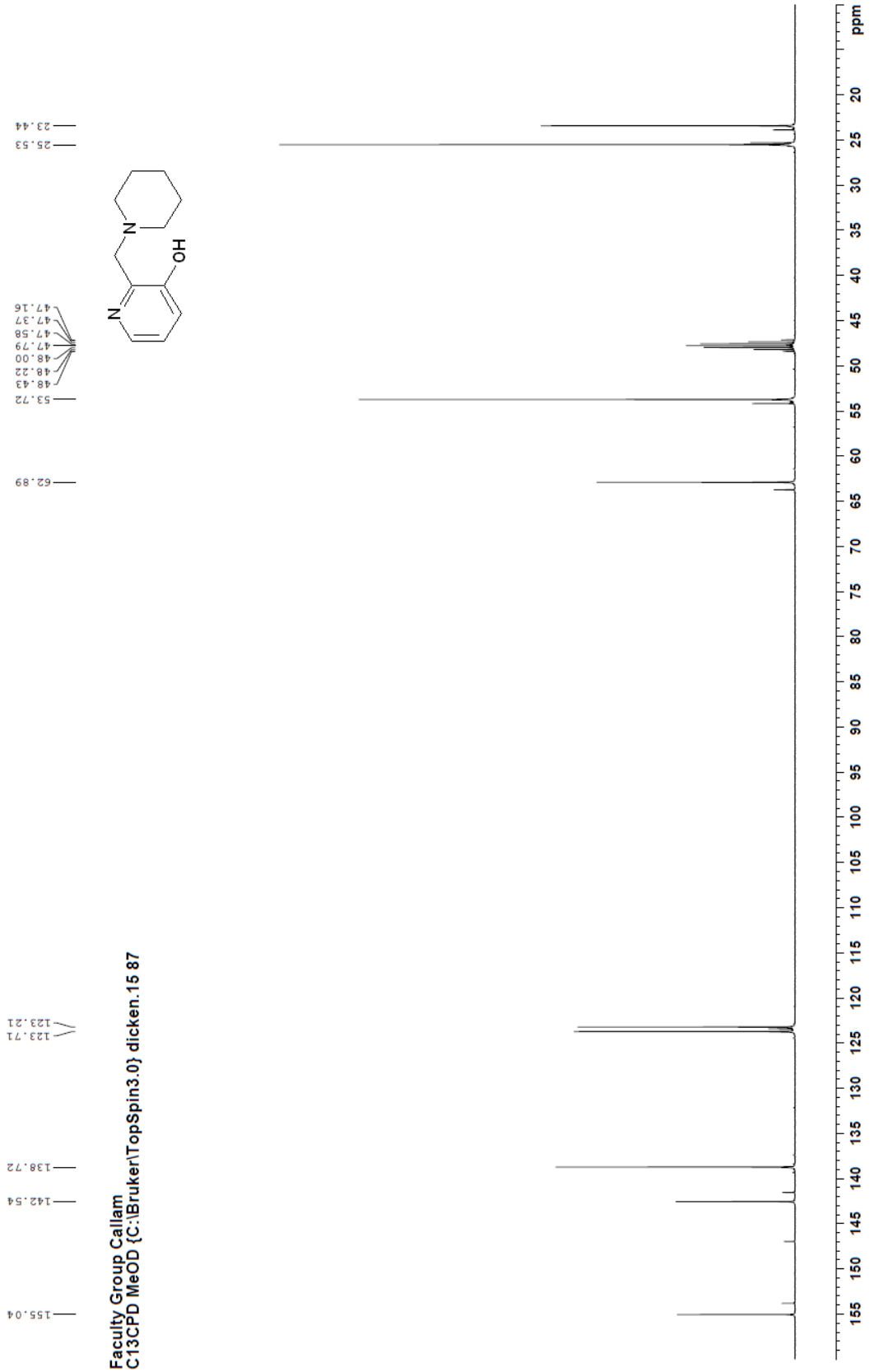
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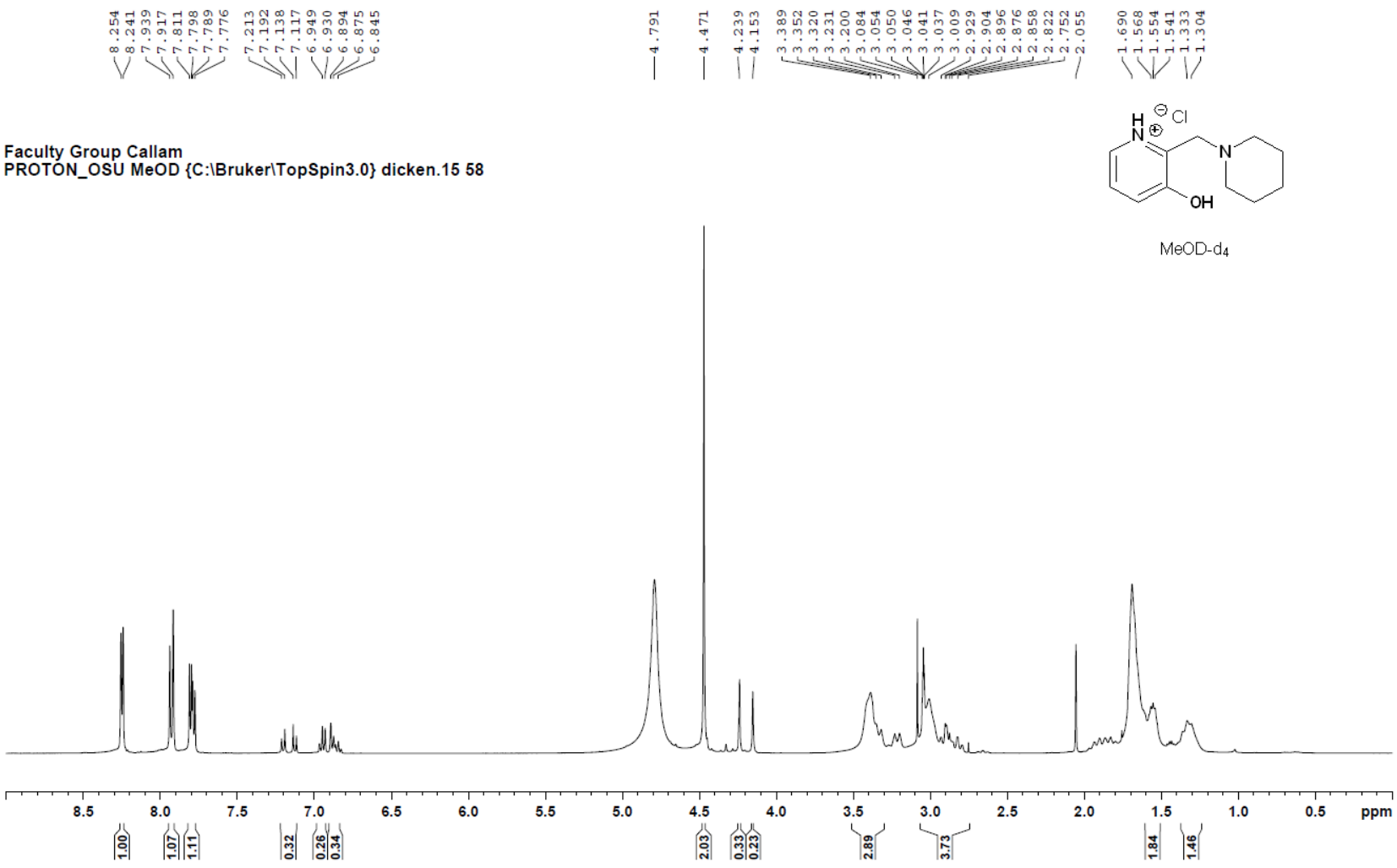
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Faculty Group Callam
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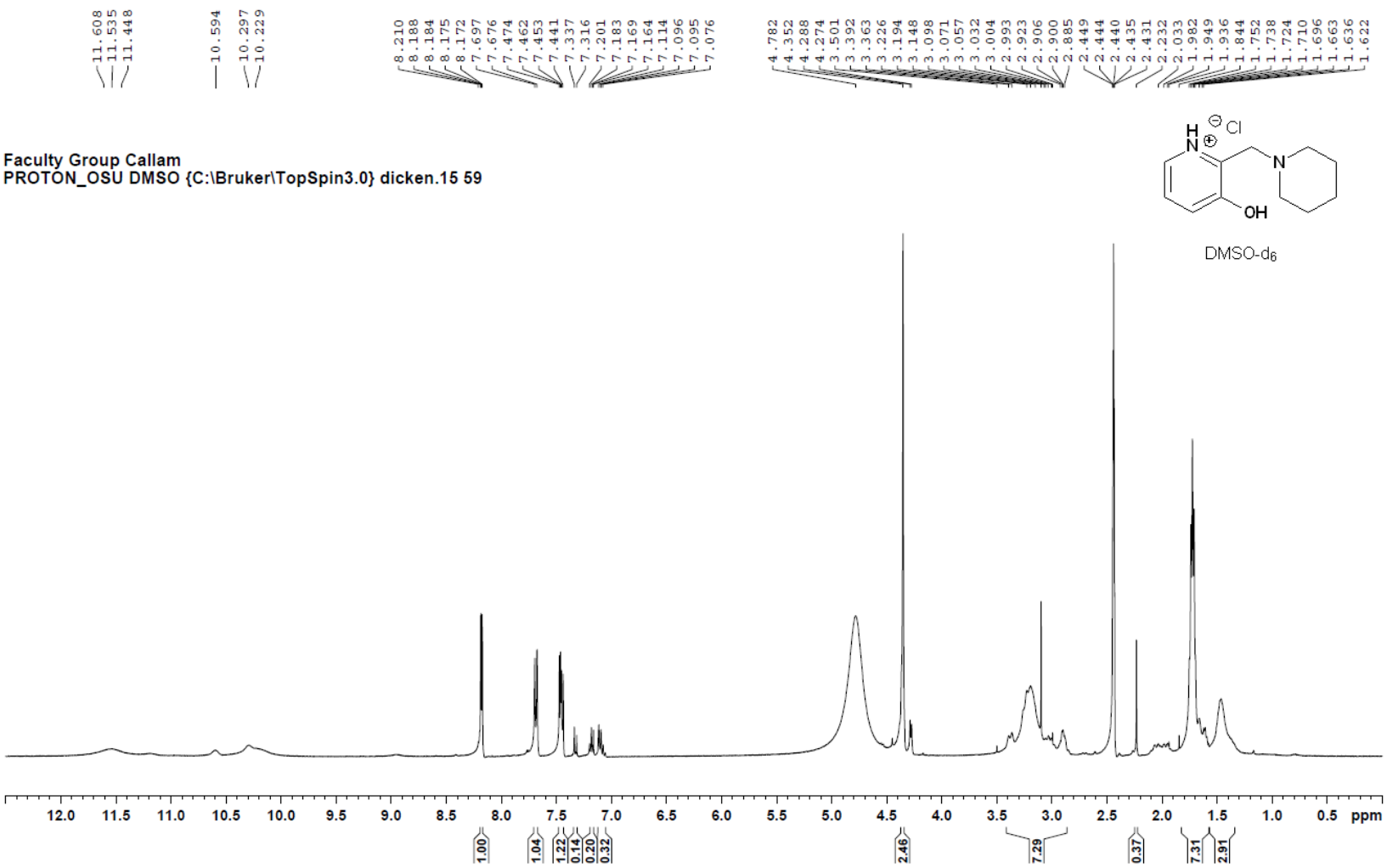
1.00
1.07
1.11
0.32
0.26
0.34
2.03
0.33
0.23
2.89
3.73
1.84
1.46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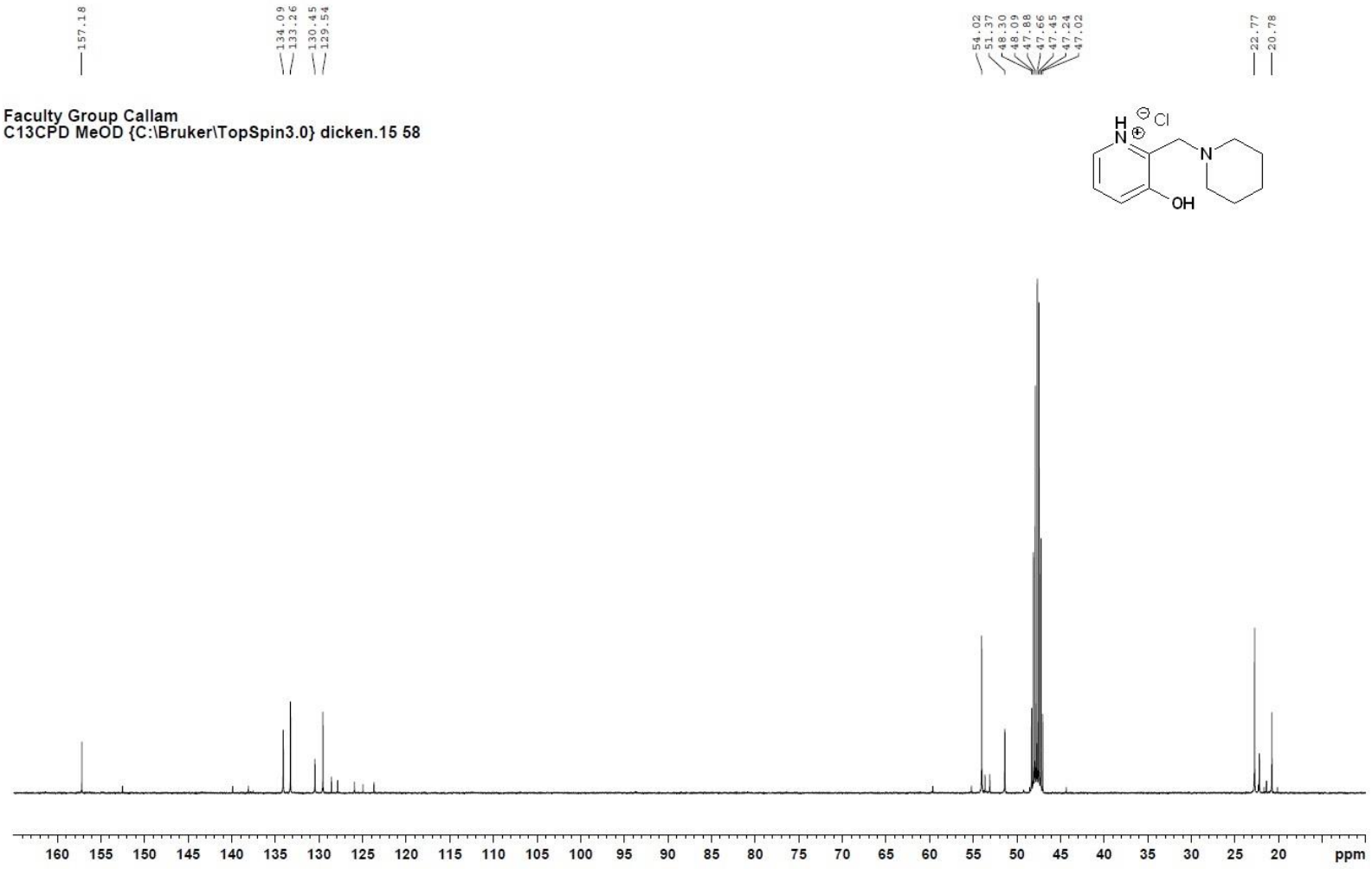
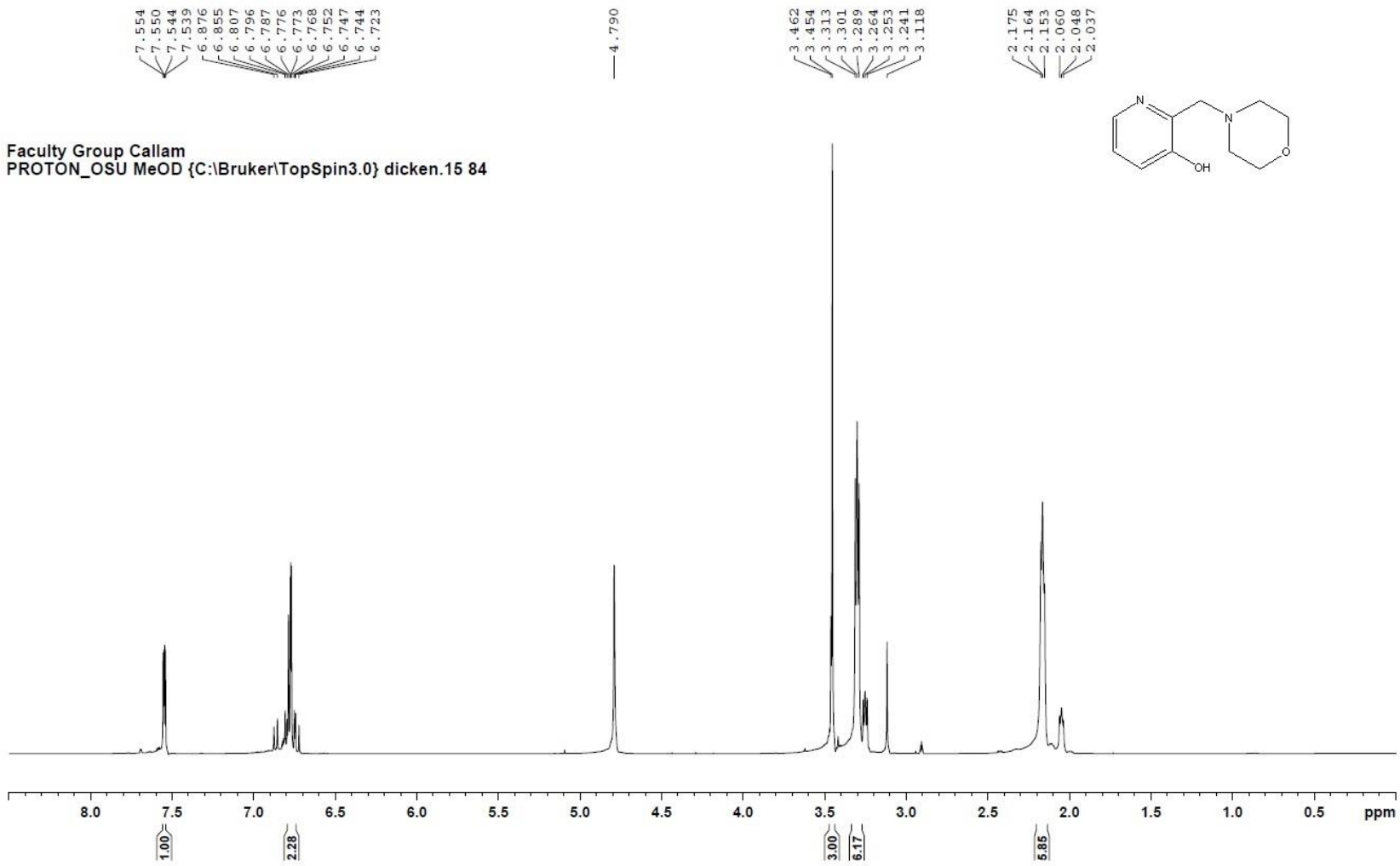
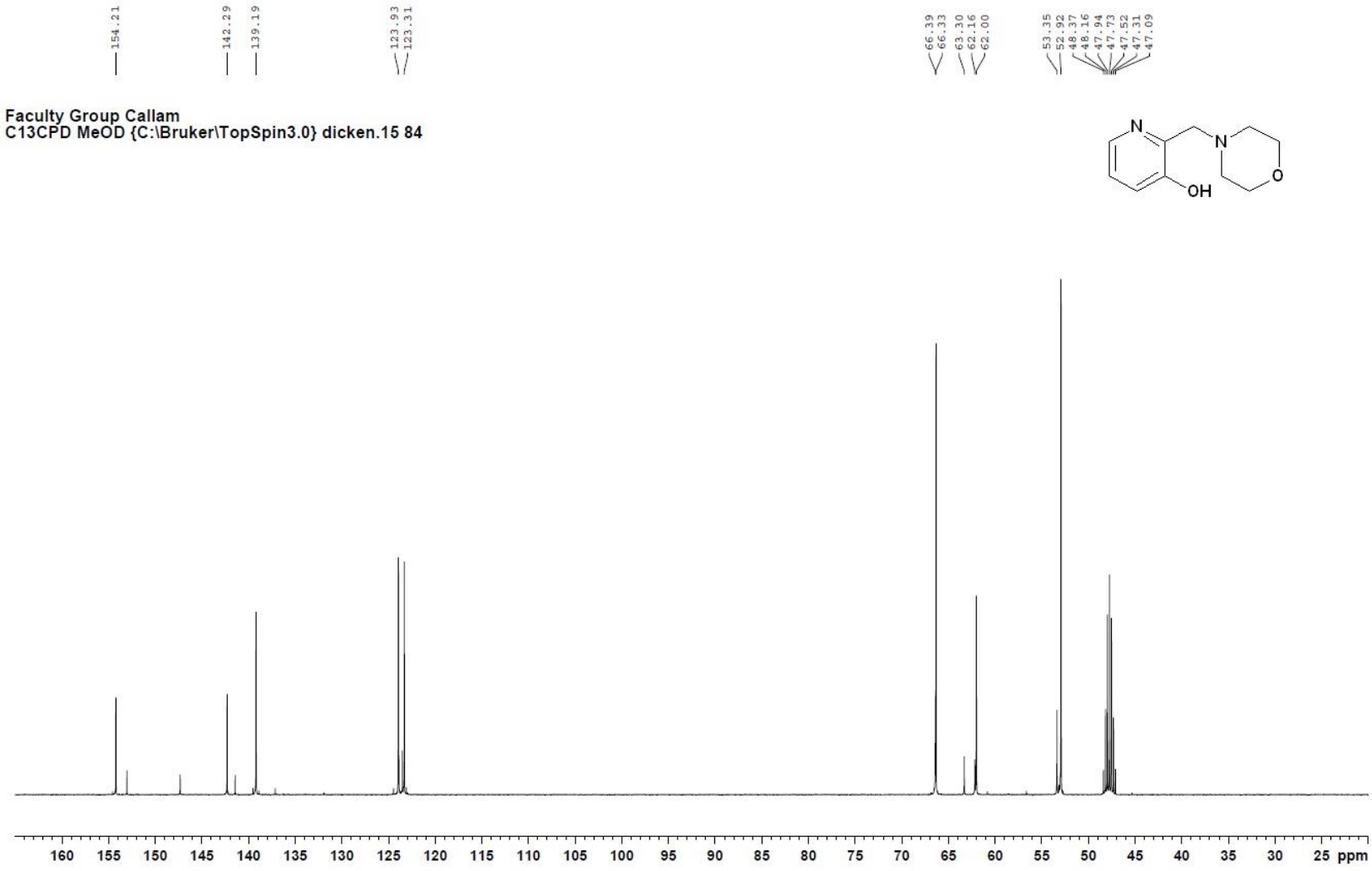
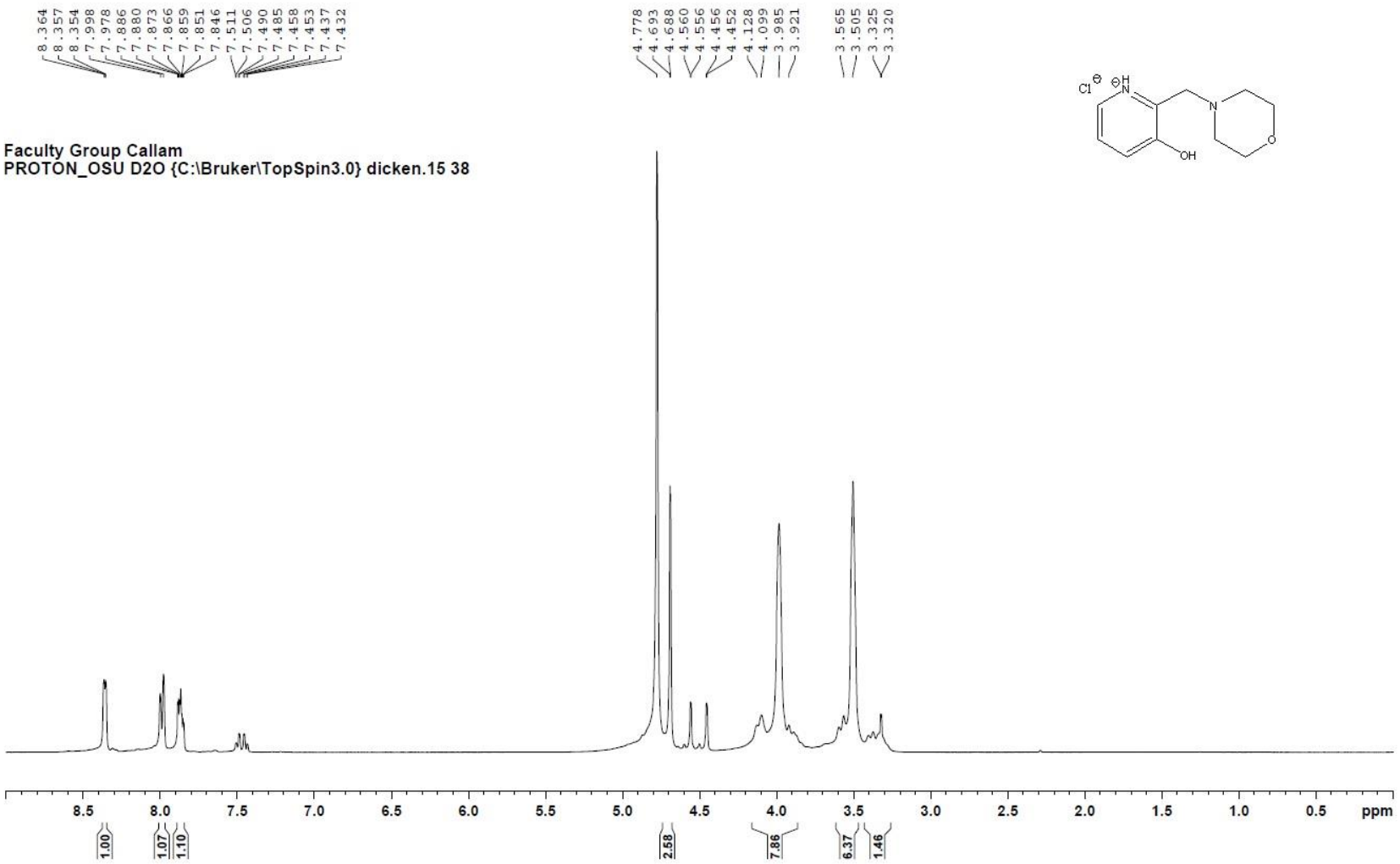
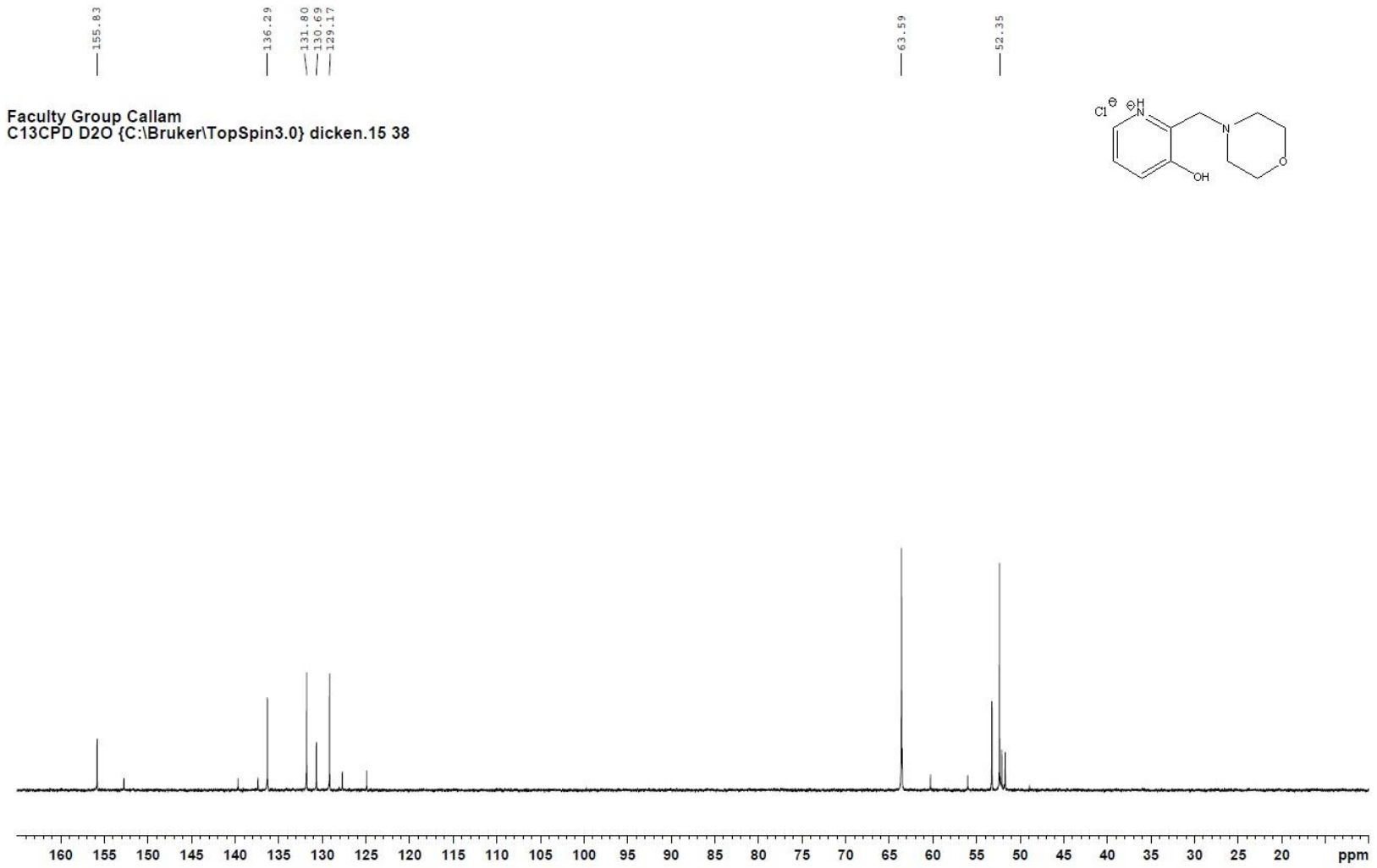
11.535
11.448
10.594
10.297
10.229
8.210
8.188
8.184
8.175
8.172
7.697
7.676
7.474
7.462
7.453
7.441
7.337
7.316
7.201
7.183
7.169
7.164
7.114
7.096
7.095
7.076
4.782
4.352
4.288
4.274
4.501
3.392
3.363
3.226
3.194
3.148
3.098
3.071
3.057
3.032
3.004
2.993
2.923
2.906
2.900
2.885
2.449
2.444
2.440
2.435
2.431
2.232
2.033
1.982
1.949
1.936
1.844
1.752
1.738
1.724
1.710
1.696
1.663
1.636
1.622
Faculty Group Callam
PROTON_OSU DMSO {C:\Bruker\TopSpin3.0} dicken.15 59
DMSO-d6
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
1.04
1.22
0.14
0.20
0.32
2.46
7.29
0.37
7.31
2.91


157.18
134.09
133.26
130.45
129.54
54.02
51.37
48.30
48.09
47.88
47.66
47.45
47.24
47.02
22.77
20.78
Faculty Group Callam
C13CPD MeOD {C:\Bruker\TopSpin3.0} dicken.15 58
Cl
OH
160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 ppm


7.554
7.550
7.544
7.539
6.876
6.855
6.807
6.796
6.787
6.776
6.773
6.768
6.752
6.747
6.744
6.723
4.790
3.462
3.454
3.313
3.301
3.289
3.264
3.253
3.241
3.118
2.175
2.164
2.153
2.060
2.048
2.037
N
N
O
OH
Faculty Group Callam
PROTON_OSU MeOD {C:\Bruker\TopSpin3.0} dicken.15 84
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
2.28
3.00
6.17
5.85


154.21
142.29
139.19
123.93
123.31
66.39
66.33
63.30
62.16
62.00
53.35
52.92
48.37
48.16
47.94
47.73
47.52
47.31
47.09
Faculty Group Callam
C13CPD MeOD {C:\Bruker\TopSpin3.0} dicken.15 84
N
N
O
OH
160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 ppm


8.364
8.357
8.354
7.998
7.978
7.886
7.880
7.873
7.866
7.859
7.851
7.846
7.511
7.506
7.490
7.485
7.458
7.453
7.437
7.432
4.778
4.693
4.688
4.560
4.556
4.456
4.452
4.128
4.099
3.985
3.921
3.565
3.505
3.325
3.320
Faculty Group Callam
PROTON_OSU D2O {C:\Bruker\TopSpin3.0} dicken.15 38
Cl
OH
N
O
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
1.07
1.10
2.58
7.86
6.37
1.46


155.83
136.29
131.80
130.69
129.17
63.59
52.35
Faculty Group Callam
C13CPD D2O {C:\Bruker\TopSpin3.0} dicken.15 38
Cl
OH
N
N
O
OH
160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 ppm